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(54) Title: IN VITRO MACROMOLECULE BIOSYNTHESIS METHODS USING EXOGENOUS AMINO ACIDS AND A NOVEL ATP REGENERATION SYSTEM		
(57) Abstract <p>Compositions and methods are provided for the enhanced <i>in vitro</i> synthesis of polypeptides. In order to improve the performance of <i>in vitro</i> protein synthesis reactions, metabolic inhibitors, or manipulation of a source organism, is used to diminish or avoid the action of enzymes responsible for undesirable amino acids production or depletion. A homeostatic system may be used for production of ATP, where the required high energy phosphate bonds are generated <i>in situ</i>, e.g. through coupling with an oxidation reaction. The homeostatic energy source will typically lack high energy phosphate bonds itself, and will therefore utilize free phosphate in the reaction mix during generation of ATP. The homeostatic energy source is provided in combination with an enzyme that catalyzes the creation of high energy phosphate bonds and with an enzyme that can use that high energy phosphate bond to regenerate ATP.</p>		

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IN VITRO MACROMOLECULE BIOSYNTHESIS METHODS USING EXOGENOUS AMINO ACIDS AND A NOVEL ATP REGENERATION SYSTEM

BACKGROUND OF THE INVENTION

The directed synthesis of proteins and other biological macromolecules is one of the great achievements of biochemistry. The development of recombinant DNA techniques has allowed the characterization and synthesis of highly purified coding sequences, which in turn can be used to produce highly purified proteins, even though in native cells the protein may be available only in trace amounts. Polypeptide chains can be synthesized by chemical or biological processes. The biological synthesis may be performed within the environment of a cell, or using cellular extracts and coding sequences to synthesize proteins *in vitro*.

For several decades, *in vitro* protein synthesis has served as an effective tool for lab-scale expression of cloned or synthesized genetic materials. In recent years, *in vitro* protein synthesis has been considered as an alternative to conventional recombinant DNA technology, because of disadvantages associated with cellular expression. *In vivo*, proteins can be degraded or modified by several enzymes synthesized with the growth of the cell, and after synthesis may be modified by post-translational processing, such as glycosylation, deamidation or oxidation. In addition, many products inhibit metabolic processes and their synthesis must compete with other cellular processes required to reproduce the cell and to protect its genetic information.

Because it is essentially free from cellular regulation of gene expression, *in vitro* protein synthesis has advantages in the production of cytotoxic, unstable, or insoluble proteins. The over-production of protein beyond a predetermined concentration can be difficult to obtain *in vivo*, because the expression levels are regulated by the concentration of product. The concentration of protein accumulated in the cell generally affects the viability of the cell, so that over-production of the desired protein is difficult to obtain. In an isolation and purification process, many kinds of protein are insoluble or unstable, and are either degraded by intracellular proteases or aggregate in inclusion bodies, so that the loss rate is high.

In vitro synthesis circumvents many of these problems. Also, through simultaneous and rapid expression of various proteins in a multiplexed configuration, this technology can provide a valuable tool for development of combinatorial arrays for research, and for screening of proteins. In addition, various kinds of unnatural amino acids can be efficiently incorporated into proteins for specific purposes (Noren *et al.* (1989) Science **244**:182-188). However, despite all its promising aspects, the *in vitro* system has not been widely accepted as a practical alternative, mainly due to the short reaction period, which causes a poor yield of protein synthesis.

The development of a continuous flow *in vitro* protein synthesis system by Spirin *et al.* (1988) Science **242**:1162-1164 proved that the reaction could be extended up to several hours. Since then, numerous groups have reproduced and improved this system (Kigawa *et al.* (1991) J. Biochem. **110**:166-168; Endo *et al.* (1992) J. Biotechnol. **25**:221-230. Recently, Kim and Choi (1996) Biotechnol. Prog. **12**: 645-649, reported that the merits of batch and continuous flow systems could be combined by adopting a 'semicontinuous operation' using a simple dialysis membrane reactor. They were able to reproduce the extended reaction period of the continuous flow system while maintaining the initial rate of a conventional batch system. However, both the continuous and semi-continuous approaches require quantities of expensive reagents, which must be increased by a significantly greater factor than the increase in product yield.

Several improvements have been made in the conventional batch system (Kim *et al.* (1996) Eur. J. Biochem. **239**: 881-886; Kuldicki *et al.* (1992) Anal. Biochem. **206**:389-393; Kawarasaki *et al.* (1995) Anal. Biochem. **226**: 320-324). Although the semicontinuous system maintains the initial rate of protein synthesis over extended periods, the conventional batch system still offers several advantages, e.g. convenience of operation, easy scale-up, lower reagent costs and excellent reproducibility. Also, the batch system can be readily conducted in multiplexed formats to express various genetic materials simultaneously.

Most recently, Patnaik and Swartz (1998) Biotechniques **24**:862-868 reported that the initial specific rate of protein synthesis could be enhanced to a level similar to that of *in vivo* expression through extensive optimization of reaction conditions. It is notable that they achieved such a high rate of protein synthesis using the conventional cell extract prepared without any condensation steps (Nakano *et al.* (1996) J. Biotechnol. **46**:275-282; Kim *et al.* (1996) Eur. J. Biochem. **239**:881-886). Kigawa *et al.* (1999) FEBS Lett **442**:15-19 report high levels of protein synthesis using condensed extracts and creatine phosphate as an energy source. These results imply that further improvement of the batch system, especially in terms of the longevity of the protein synthesis reaction, would substantially increase the productivity for batch *in vitro* protein synthesis. However, the reason for the early halt of protein synthesis in the conventional batch system has remained unclear.

As shown from the above, both protein productivity and production amount are still low, which is an obstacle in implementing the industrialization of cell-free protein synthesis. Therefore, improvements are greatly required in terms of the total productivity of the protein by increasing the specific production rate and the length of system operation. Optimizing these conditions is of great interest for development of commercial processes.

Relevant literature

Muller *et al.* (1993) Science 259:965-967 describes the structure of the thiamine- and flavin- dependent enzyme, pyruvate oxidase. Ryabova *et al.* (1995) Anal. Biochem. 226:184-186 describe the use of acetyl phosphate as an energy source for bacterial cell-free translation systems. Pyruvate oxidase mutants are described in U.S. Patent no. 5,153,138, and pyruvate oxidase is further described in U.S. Patent no. 4,666,832 and 4,246,342.

SUMMARY OF THE INVENTION

Compositions and methods are provided for the enhanced *in vitro* synthesis of biological molecules. Of particular interest is the synthesis of polymers, *e.g.* nucleic acids, polypeptides, and complex carbohydrates where ATP is required for synthesis. In one embodiment of the invention, a homeostatic system is used for production of ATP, where use of this energy system prevents a net increase in free phosphate as a result of ATP hydrolysis. The phosphate that is released from ATP hydrolysis is recycled into high energy phosphate bonds, thereby preventing its accumulation. Energy homeostasis is accomplished by addition of a regenerative energy source, *e.g.* pyruvate in combination with the enzyme pyruvate oxidase to generate acetyl phosphate. In a preferred embodiment, energy sources such as phosphoenol pyruvate, which increase the net free phosphate in the reaction mix, are omitted.

In another embodiment of the invention, *in vitro* synthesis of protein molecules is enhanced by optimizing the metabolism of amino acids present in the reaction mix. The optimal amino acid concentration is maintained by inhibiting enzymes that act to undesirably metabolize specific amino acids. The concentration of certain amino acids, including tryptophan, cysteine and arginine, has been found to decrease during conventional reactions due to degradation reactions. Other amino acids, including alanine, aspartate and asparagine, undesirably increase in concentration at the expense of the provided energy source. Inhibition of enzymes catalyzing these reactions is achieved by addition of inhibitory compounds to the reaction mix; modification of the reaction mixture to decrease or eliminate the responsible enzyme activities; or a combination of the two.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the effects of adding amino acids and energy source after pre-incubation.

Figure 2 is a time course showing the effects of adding pyruvate and amino acids.

Figures 3A and 3B show the changes of amino acid concentrations during *in vitro* protein synthesis.

Figure 4 shows the effects of supplementing synthetic reactions with specific amino acids.

5 Figure 5 shows the effect of spermidine on arginine depletion

Figure 6 shows the effect of oxalic acid on asx generation.

Figure 7 is a time course of protein synthesis using pyruvate as an energy source, with or without oxalic acid.

10 Figure 8 is a time course of protein synthesis using phosphoenol pyruvate as an energy source, with or without oxalic acid.

Figure 9 illustrates the metabolic pathways of pyruvate in *E. coli*.

Figure 10 illustrates the metabolic pathways leading to the formation of putrescine and spermidine.

15 Figures 11A and 11B are flow diagrams illustrating regeneration of ATP in an *in vitro* protein synthesis system. Fig. 11A depicts a conventional scheme using phosphoenol pyruvate (PEP) and pyruvate kinase (PK); Fig. 11B depicts the use of pyruvate, pyruvate oxidase (Pox) and endogenous acetyl kinase (Ack).

Figure 12 is a graph showing accumulation of inorganic phosphate in the reaction mixture under various conditions.

20 Figure 13 is a graph depicting the use of pyruvate as energy source for protein synthesis.

Figure 14 is a time course of CAT synthesis in the conventional system using PEP (open circles) and using pyruvate (closed circles).

25 Figure 15 is a time course of CAT mRNA concentration during the incubation of PEP(open circle) and pyruvate (closed circle).

Figure 16 is an SDS-PAGE analysis of *in vitro* synthesized CAT.

Figure 17 shows the expression of proteins using PEP or pyruvate as energy sources.

30 Figure 18 is a graph depicting the different sensitivity of CAT and hLT production to the concentrations of exogenous inorganic phosphate and magnesium ion.

Figure 19A and Figure 19B depict the effect of repeated addition of the energy source.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Compositions and methods are provided for the enhanced *in vitro* synthesis of protein molecules, by optimizing the metabolism of amino acids present in the reaction mix and/or the use of a regenerative energy system. The concentration of certain amino acids, including tryptophan, cysteine and arginine, has been found to decrease during conventional reactions due to degradation reactions. Other amino acids, including alanine, aspartate and asparagine, undesirably increase in concentration at the expense of the provided energy source. Reducing the enzymatic activities responsible for catalyzing these reactions improves the overall yield of synthesis.

The key ingredients for a cell-free protein synthesis system are a continuous ATP supply, an adequate amino acid supply and active catalysts. During the performance of a typical *in vitro* protein synthesis reaction utilizing microbial extracts, it was discovered that addition of more energy source by itself did not significantly prolong the duration of protein synthesis. It was then determined that reaction mixtures lost their synthetic ability during pre-incubation, even in the absence of active protein synthesis. Addition of more energy source was not sufficient to restore synthetic activity, which required the addition of amino acids as well. Addition of both these components did, in fact, allow a substantial extension of the reaction period for protein synthesis, with a coordinate increase in protein yield.

The concentration of some amino acids was found to increase significantly during either pre-incubation or protein synthesis, while others were being depleted. In particular, when pyruvate was being used as the energy source, alanine, aspartic acid and/or asparagine significantly increased in concentration. Arginine, cysteine and tryptophan decreased to the point of depletion. The length of active synthesis can be extended by the addition of energy source and just these three amino acids.

In order to improve the performance of *in vitro* protein synthesis reactions, metabolic inhibitors, or manipulation of a source organism, is used to decrease the action of enzymes responsible for undesirable amino acids production or depletion.

Either performed alone, or in combination with optimized amino acid sources, compositions and methods are provided for a homeostatic system used for production of ATP, where use of this energy system prevents a net increase in free phosphate as a result of ATP hydrolysis. The phosphate that is released from ATP hydrolysis is recycled into the energy pool, thereby preventing its accumulation.

The primary source of energy for most biological synthesis is ATP. ATP is generated from ADP and a secondary source of high energy phosphate bonds, e.g. phosphoenol pyruvate (PEP), creatine phosphate, or acetyl phosphate. These chemicals recharge ADP

into ATP by catalysis with the enzymes pyruvate kinase, creatine kinase, and acetate kinase, respectively. In addition, there are some reports regarding the use of polyphosphate to recycle ATP. Energy homeostasis is accomplished by addition of a secondary energy source that indirectly provides high energy phosphate bonds without release of free phosphate.

Exemplary is the use of pyruvate in combination with the enzyme pyruvate oxidase as the homeostatic energy source. During oxidation of pyruvate, acetyl phosphate is generated, which then directly regenerates ATP from ADP. The metabolic pathway is shown in Figure 11B. The phosphate that is hydrolyzed from ATP is recycled during the pyruvate oxidation to generate acetyl phosphate, thereby preventing a net accumulation of free phosphate, which can have an inhibitory effect on synthetic reactions. The reaction mix may comprise small amounts of secondary energy sources that result in free phosphate accumulation, e.g. acetyl phosphate, PEP, creatine phosphate, but at a significantly lower concentration than with previously described *in vitro* synthesis reactions.

While the present invention has been described primarily in terms of synthetic reactions, one of skill in the art will appreciate the use of the subject homeostatic energy system in other *in vitro* reactions. For example, protein folding and protein transport require the use of ATP, and may benefit from the subject methods.

DEFINITIONS

It is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, and reagents described, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

As used herein the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the protein" includes reference to one or more proteins and equivalents thereof known to those skilled in the art, and so forth. All technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs unless clearly indicated otherwise.

In vitro synthesis: as used herein refers to the cell-free synthesis of biological macromolecules, e.g. polypeptides, in a reaction mix comprising biological extracts and/or defined reagents. The reaction mix will comprise at least ATP, an energy source; a template

for production of the macromolecule, e.g. DNA, mRNA, etc.; amino acids, and such co-factors, enzymes and other reagents that are necessary for the synthesis, e.g. ribosomes, tRNA, polymerases, transcriptional factors, etc. In one embodiment of the invention, the energy source is a homeostatic energy source. Also included may be enzyme(s) that catalyze the regeneration of ATP from high energy phosphate bonds, e.g. acetate kinase, creatine kinase, etc. Such enzymes may be present in the extracts used for translation, or may be added to the reaction mix. Such synthetic reaction systems are well-known in the art, and have been described in the literature. The cell free synthesis reaction may be performed as batch, continuous flow, or semi-continuous flow, as known in the art.

Enhanced reaction mix: as used herein refers to a reaction mixture capable of catalyzing the synthesis of polypeptides from a nucleic acid template; and which has been optimized to maintain the concentration of specific amino acids during pre-incubation and active protein synthesis. The mixture may comprise metabolic inhibitors that decrease undesirable enzymatic reactions. Alternatively, or in combination, the enhanced reaction mix will be engineered through genetic or other processes to decrease the enzymatic activity responsible for undesirable side-reactions, that result in amino acid depletion or accumulation. Specific optimization conditions are provided below.

The particular improvements are described separately, i.e. enhanced conditions for tryptophan metabolism, enhanced conditions for alanine metabolism, etc. However, it will be understood by one of skill in the art that these improved conditions may be used in various combinations, where inhibitors for one set of reaction are present in a reaction mix depleted for undesirable enzymes, and the like.

In a preferred embodiment of the invention, the reaction mixture comprises extracts from bacterial cells, e.g. *E. coli* S30 extracts, as is known in the art. For convenience, the organism used as a source of extracts may be referred to as the source organism. While such extracts are a useful source of ribosomes and other factors necessary for protein synthesis, they can also contain small amounts of enzymes responsible for undesirable side-reactions that are unrelated to protein synthesis, but which deplete amino acids, ATP, pyruvate or other reagents.

Methods for producing active extracts are known in the art, for example they may be found in Pratt (1984), coupled transcription-translation in prokaryotic cell-free systems, p. 179-209, in Hames, B. D. and Higgins, S. J. (ed.), Transcription and Translation: a practical approach, IRL Press, New York. Kudlicki et al. (1992) Anal Biochem 206(2):389-93 modify

the S30 *E. coli* cell-free extract by collecting the ribosome fraction from the S30 by ultracentrifugation.

The extracts may be optimized for expression of genes under control of a specific promoter, (for example see Nevin and Pratt (1991) FEBS Lett **291**(2):259-63, which system consists of an *E. coli* crude extract (prepared from cells containing endogenous T7 RNA polymerase) and rifampicin (an *E. coli* RNA polymerase inhibitor)). Kim *et al.* (1996) Eur. J. Biochem. **239**: 881-886 further enhance protein production by optimizing reagent concentrations.

The enhanced reaction mix may comprise metabolic inhibitors of the undesirable enzyme activity. Frequently such inhibitors will be end-products of the reaction, that then inhibit by a feedback mechanism. The specific inhibitors are determined based on the metabolic pathways of the source organism. These pathways are well-known in the art for many bacterial and eukaryotic species, *e.g. E. coli, S. cerevisiae, H. sapiens, etc.* The inhibitor is added at a concentration sufficient to inhibit the undesirable enzymatic activity while increasing protein synthesis.

Pathways of particular interest relate to the metabolism of pyruvate in *E. coli* cells shown in Figure 9, including the production of alanine from pyruvate by a transaminase reaction with glutamic acid; and synthesis of aspartate from oxalacetate. Other pathways of interest relate to the degradation of amino acids, for example the degradation of arginine to form putrescine and spermidine, shown in Figure 10.

In an alternative embodiment to adding metabolic inhibitors, the undesirable enzymes may be removed or otherwise deleted from the reaction mix. In one embodiment of the invention, the coding sequence for the enzyme is "knocked-out" or otherwise inactivated in the chromosome of the source organism, by deletion of all or a part of the coding sequence; frame-shift insertion; dominant negative mutations, *etc.* The genomes of a number of organisms, including *E. coli*, have been completely sequenced, thereby facilitating the genetic modifications. For example, a markerless knockout strategy method is described by Arigoni *et al.* (1998) Nat Biotechnol **16**(9):851-6.

A preferred method for inactivating targeted genes is described by Hoang *et al.* (1998) Gene **212**:77-86. In this method, gene replacement vectors are employed that contain a tetracycline resistance gene and a gene encoding levan sucrase (*sacB*) as selection markers for recombination. The target gene is first cloned and mutagenized, preferably by deleting a significant portion of the gene. This gene is then inserted by ligation into a vector designed for facilitating chromosomal gene replacement. The *E. coli* cells are then transformed with those vectors. Cells that have incorporated the plasmid into the

chromosome at the site of the target gene are selected, then the plasmid is forced to leave the chromosome by growing the cells on sucrose. Sucrose is toxic when the *sacB* gene resides in the chromosome. The properly mutated strain is selected based on its phenotype of tetracycline sensitivity and sucrose resistance. PCR analysis or DNA sequencing then
5 confirms the desired genetic change.

However, in some cases the enzyme reducing the duration and yield of the protein synthesis reaction may be essential for the growth of the source organism. In those cases, a conditional knock-out may be used. For example, anti-sense sequences corresponding to the targeted gene are introduced into the source organism on an inducible promoter. The
10 cells are grown for a period of time, and then the anti-sense construct induced, in order to deplete the cell of the targeted enzyme.

The enzyme can be removed from the cell extract after cell disruption and before use. Any of the several means known in the art of protein purification may be used, including affinity purification techniques such as the use of antibodies or antibody fragments
15 with specific affinity for the target enzymes; use of affinity tags expressed as part of the target enzymes to facilitate their removal from the cell extract; and conventional purification methods.

In another embodiment, an antibody or antibody fragment (e.g., Fab or scFv) is selected for specific affinity for the target enzyme using phage display or other well
20 developed techniques. That antibody or antibody fragment is then immobilized on any of several purification beads or resins or membranes using any of several immobilization techniques. The immobilized antibody is contacted with the cell extract to bind to the target enzyme, and the immobilized antibody/enzyme complex then removed by filtration or gentle centrifugation.

For example, the coding sequence of the targeted protein may be modified to include
25 a tag, such as the Flag® extension (developed by Immunex Corp. and sold by Stratagene), or a poly-histidine tail. Many other examples have been published and are known to those skilled in the art. The tagged proteins are then removed by passage over the appropriate affinity matrix or column. The amino acid extension and binding partner are chosen so that
30 only specific binding occurs under conditions compatible with the stability of the cell extract, and without significantly altering the chemical composition of the cell extract.

In yet another example, the target enzyme or enzymes are separated by any of several methods commonly used for protein purification, such as substrate affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography,
35 electrophoretic separation, or other methods practiced in the art of protein purification.

Tryptophan/Cysteine optimized reaction mix: The concentration of both tryptophan and cysteine is observed to decrease rapidly during pre-incubation or protein synthesis. The optimized reaction mix for these amino acids is deficient in tryptophanase activity. The tryptophanase gene (tnaA) sequence of *E. coli* may be found in Deely and Yanofsky (1981) J. Bact. **147**: 787-796; Genbank accession no. 1790144; locus AE000448, accession AE000448 of *E. coli* complete genome sequence. Using publicly available genetic sequences, the activity of the tryptophanase may be inactivated in a modified bacterial cell, as described above. Alternatively, the enzyme activity is depleted from the reaction mix by affinity purification, or conventional purification methods.

Arginine optimized reaction mix: Arginine is also depleted from the synthetic reaction mix, at least in part due to the conversion of arginine to putrescine and spermidine. This is one of two known pathways for putrescine and spermidine production. The first enzyme in this pathway is arginine decarboxylase. This enzyme is known to be feedback inhibited by spermidine (Tabor and Tabor (1969) J. Biol. Chem. **244**: 2286-2292). Arginine optimized reactions may therefore comprise a metabolic inhibitor of this pathway, or may be modified to decrease the arginine decarboxylase activity.

In one embodiment of the invention, spermidine is added as an inhibitor at an initial concentration of at least about 0.5 mM and not more than about 500 mM, usually at least about 1 mM, and preferably at about 5 mM concentration. Other products of this pathway include putrescine and agmatine.

In another embodiment, the arginine decarboxylase (speA) gene of *E. coli* is inactivated. The genetic sequence may be accessed through Genbank, no. 1789307; locus AE000377, accession AE000377. The complete chromosomal sequence of *E. coli* has been published in Blattner et al. (1997) *Science* **277**:1453-1474. As described above, the gene may be inactivated, or the protein product otherwise depleted from the reaction mixture.

Depletion of this enzyme may also be accomplished by growing cells in the absence of arginine to force the cell to make putrescine and spermidine from ornithine using an alternate pathway. The activity of arginine decarboxylase in such a cell is significantly lower.

Asx optimized reaction mix: Aspartic acid and asparagine are formed from phosphoenol pyruvate. The enzyme phosphoenol pyruvate synthetase (pps) converts pyruvate into PEP and consumes 2 equivalents of high-energy phosphate bonds (as ATP is converted to AMP) per molecule of PEP synthesized. When pyruvate is being used as an

energy source, this enzyme therefore has the potential to waste both pyruvate and ATP, thereby robbing the protein synthesis reaction of its energy supply.

Addition of oxalic acid, which has been reported to inhibit pps (Narindrasorasak and Bridger (1978) *Can. J. Biochem.* **56**: 816-9), was able to extend the reaction period both in the PEP and pyruvate systems. With both pyruvate and PEP as energy sources, inhibiting pps with oxalic acid decreased the rate of asp/asn production and increased the protein yield. Oxalic acid is added at a concentration of at least about 0.5 mM, and not more than about 100 mM, usually at least about 1 mM, and preferably at a concentration of about 3 mM.

For efficient use of energy source in both the PEP and the pyruvate system, the genes for *E. coli* pyruvate oxidase, which converts pyruvate into acetate consuming oxygen, and/or phosphoenol pyruvate synthetase (pps) can be disrupted or otherwise inactivated. The coding sequence for *E. coli* phosphoenol pyruvate synthetase may be accessed in Genbank, no. X59381; and is also published in Niersbach *et al.* (1992) Mol. Gen. Genet. **231**:332-336. The coding sequence for *E. coli* pyruvate oxidase may be accessed in Genbank, no. X04105; and is also published in Grabau and Cronan (1986) Nucleic Acids Res. **14**:5449-5460.

Alanine optimized reaction mix: Alanine has been found to accumulate in synthetic reactions, at least in part because a conversion of pyruvate to alanine through an alanine-glutamate transaminase. This enzyme is described in Wang *et al.* (1987) J. Bacteriol **169**(12):5610-4. As described above, this gene may be isolated as described by Wang *et al.*, sequenced and then inactivated; or the enzymatic activity otherwise depleted from the reaction mix.

Homeostatic energy source, as used herein, refers to a secondary source of energy for driving *in vitro* synthetic reactions utilizing ATP as a primary energy source, whereby the free phosphate generated by ATP hydrolysis is recycled. Instead of exogenous addition of a source of high energy phosphate bonds, the required high energy phosphate bonds are generated *in situ*, e.g. through coupling with an oxidation reaction. The homeostatic energy source will typically lack high energy phosphate bonds itself, and will therefore utilize free phosphate present in the reaction mix during ATP regeneration. Since inorganic phosphate can be an inhibitory by-product of synthesis, the period of time when synthesis is maintained *in vitro* can be extended. The homeostatic energy source is provided in combination with an enzyme that catalyzes the creation of high energy phosphate bonds.

In one embodiment of the invention, the homeostatic energy source is pyruvate, which may be supplied as a suitable biologically acceptable salt, or as the free acid, pyruvic acid. The final concentration of pyruvate at initiation of synthesis will usually be at least about 1 mM, more usually at least about 10 mM, and not more than about 500 mM, usually not more than about 100 mM. Additional pyruvate may be added to the reaction mix during the course of synthesis to provide for longer reaction times.

Preferably, secondary energy sources that result in the net accumulation of free phosphate, including creatine phosphate, polyphosphate, acetyl phosphate and phosphoenol pyruvate, are not added to the reaction mix at a significant concentration. The concentration of such energy sources in the reaction mix at the initiation of synthesis will generally be less than about 10 mM, more usually less than about 1 mM, and may be less than about 0.5 mM. Excluded from such energy sources is ATP itself, which will be included in the reaction mix at conventional concentrations as a primary energy source.

Regenerative enzyme: as herein refers to an enzyme that regenerates a high energy phosphate bond from free phosphate and the homeostatic energy source, where the free phosphate may be recycled from hydrolysis of ATP. Where the homeostatic energy source is pyruvate, the enzyme will catalyze the formation of acetyl phosphate from pyruvate, as shown in Figure 11B. Since acetyl phosphate can be used as an energy source for synthesis, the pyruvate oxidase reaction provides the basis for regeneration of ATP that has been hydrolyzed in the process of protein synthesis.

An exemplary regenerative enzyme is pyruvate oxidase, EC 1.2.3.3.; CAS: 9001-96-1. It is known that pyruvate oxidase is produced by a variety of microorganisms. For example, it is known to be produced by *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, microorganisms of the genus *Pediococcus*, *Streptococcus*, and *Aerococcus*, microorganisms of the genus *Leuconostoc*, etc.

The reaction mix will comprise a concentration of regenerative enzyme sufficient to maintain the ATP pool, usually at least about 0.1 U/ml, more usually at least about 1 U/ml, and preferably at least about 5 U/ml, where the unit definition is that one unit will produce 1.0 μ mole of H_2O_2 per minute during the conversion of pyruvate and phosphate to acetylphosphate and CO_2 . It will be understood by one of skill in the art that higher concentration may be present, although generally at less than about 1000 U/ml.

The regenerative enzyme may be provided for in the reaction mix in a variety of ways. Purified or semi-purified enzyme may be added to the reaction mix. Commercial preparations of pyruvate oxidase are available, or the enzyme may be purified from natural

or recombinant sources according to conventional methods. The genetic sequences of pyruvate oxidases may be used as a source of recombinant forms of the enzyme, for example see Muller *et al.*, *supra.*; Pellerberg *et al.* (1996) Mol. Microbiol. 19:803-813; Zhou *et al.*, Genbank accession number 3818594, *etc.*

5 The enzyme may also be included in the extracts used for synthesis. For example, extracts can be derived from *E. coli* for protein synthesis. The *E. coli* used for production of the extracts may be genetically modified to encode a suitable pyruvate oxidase. Alternatively, where the synthetic reactions are protein synthesis, a template, e.g. mRNA encoding pyruvate oxidase, plasmid comprising a suitable expression construct of pyruvate
10 oxidase, *etc.* may be spiked into the reaction mix, such that a suitable amount of pyruvate oxidase is produced during synthesis.

METHODS FOR ENHANCED *IN VITRO* SYNTHESIS

The subject synthetic systems may be used separately or in combination for *in vitro*
15 reactions. The methods of the invention find use for *in vitro* synthetic reactions, particularly polypeptide synthetic reactions, or reactions that utilize ATP as a primary energy source, particularly where the accumulation of free phosphate is inhibitory to the reaction.

The methods utilizing a regenerative energy source are also applicable to such *in vitro* systems as protein folding, protein transport, *etc.* Synthetic systems of interest include
20 the replication of DNA, protein synthesis, which may include amplification of the DNA, the transcription of RNA from DNA or RNA templates, the translation of RNA into polypeptides, and the synthesis of complex carbohydrates from simple sugars. templates. The reactions may utilize a large scale reactor, small scale, or may be multiplexed to perform a plurality of simultaneous syntheses. Continuous reactions will use a feed mechanism to introduce a
25 flow of reagents, and may isolate the end-product as part of the process. Batch systems are also of interest, where additional reagents may be introduced to prolong the period of time for active synthesis. A reactor may be run in any mode such as batch, extended batch, semi-batch, semi-continuous, fed-batch and continuous, and which will be selected in accordance with the application purpose.

30 Of particular interest is the translation of mRNA to produce proteins, which translation may be coupled to *in vitro* synthesis of mRNA from a DNA template. Such a cell-free system will contain all factors required for the translation of mRNA, for example ribosomes, amino acids, tRNAs, aminoacyl synthetases, elongation factors and initiation factors. Cell-free systems known in the art include wheat germ extracts (Roberts *et al.* (1973) P.N.A.S.
35 70:2330), reticulocyte extracts (Pelham *et al.* (1976) Eur. J. Biochem. 67:247), *E. coli*

extracts, *etc.*, which can be treated with a suitable nuclease to eliminate active endogenous mRNA.

In addition to the above components such as cell-free extract, genetic template, amino acids and energy sources, materials specifically required for protein synthesis may be added to the reaction. These materials include salt, polymeric compounds, cyclic AMP, inhibitors for protein or nucleic acid degrading enzymes, inhibitor or regulator of protein synthesis, oxidation/reduction adjuster, non-denaturing surfactant, buffer component, spermine, spermidine, *etc.*

The salts preferably include potassium, magnesium, ammonium and manganese salt of acetic acid or sulfuric acid, and some of these may have amino acids as a counter anion. The polymeric compounds may be polyethylene glycol, dextran, diethyl aminoethyl, quaternary aminoethyl and aminoethyl. The oxidation/reduction adjuster may be dithiothreitol, ascorbic acid, glutathione and/or their oxides. Also, a non-denaturing surfactant such as Triton X-100 may be used at a concentration of 0-0.5 M. Spermine and spermidine may be used for improving protein synthetic ability, and cAMP may be used as a gene expression regulator.

When changing the concentration of a particular component of the reaction medium, that of another component may be changed accordingly. For example, the concentrations of several components such as nucleotides and energy source compounds may be simultaneously controlled in accordance with the change in those of other components. Also, the concentration levels of components in the reactor may be varied over time.

Preferably, the reaction is maintained in the range of pH 5-10 and a temperature of 20°-50° C., and more preferably, in the range of pH 6-9 and a temperature of 25°-40° C.

When using a protein isolating means in a continuous operation mode, the product output from the reactor flows through a membrane into the protein isolating means. In a semi-continuous operation mode, the outside or outer surface of the membrane is put into contact with predetermined solutions that are cyclically changed in a predetermined order. These solutions contain substrates such as amino acids and nucleotides. At this time, the reactor is operated in dialysis, diafiltration batch or fed-batch mode. A feed solution may be supplied to the reactor through the same membrane or a separate injection unit. Synthesized protein is accumulated in the reactor, and then is isolated and purified according to the usual method for protein purification after completion of the system operation.

Where there is a flow of reagents, the direction of liquid flow can be perpendicular and/or tangential to a membrane. Tangential flow is effective for recycling ATP and for

preventing membrane plugging and may be superimposed on perpendicular flow. Flow perpendicular to the membrane may be caused or effected by a positive pressure pump or a vacuum suction pump. The solution in contact with the outside surface of the membrane may be cyclically changed, and may be in a steady tangential flow with respect to the membrane. The reactor may be stirred internally or externally by proper agitation means.

During protein synthesis in the reactor, the protein isolating means for selectively isolating the desired protein may include a unit packed with particles coated with antibody molecules or other molecules immobilized with a component for adsorbing the synthesized, desired protein, and a membrane with pores of proper sizes. Preferably, the protein isolating means comprises two columns for alternating use. Alternately, the protein product may be absorbed using expanded bed chromatography, in which case a membrane may or may not be used.

The amount of protein produced in a translation reaction can be measured in various fashions. One method relies on the availability of an assay which measures the activity of the particular protein being translated. An example of an assay for measuring protein activity is a luciferase assay system, or chloramphenicol acetyl transferase assay system. These assays measure the amount of functionally active protein produced from the translation reaction. Activity assays will not measure full length protein that is inactive due to improper protein folding or lack of other post translational modifications necessary for protein activity.

Another method of measuring the amount of protein produced in coupled *in vitro* transcription and translation reactions is to perform the reactions using a known quantity of radiolabeled amino acid such as ³⁵S-methionine or ³H-leucine and subsequently measuring the amount of radiolabeled amino acid incorporated into the newly translated protein. Incorporation assays will measure the amount of radiolabeled amino acids in all proteins produced in an *in vitro* translation reaction including truncated protein products. The radiolabeled protein may be further separated on a protein gel, and by autoradiography confirmed that the product is the proper size and that secondary protein products have not been produced.

It is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, constructs, and reagents described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing, for example, the cell lines, constructs, and methodologies that are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the subject invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to ensure accuracy with respect to the numbers used (e.g. amounts, temperature, concentrations, etc.) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees centigrade; and pressure is at or near atmospheric.

EXPERIMENTAL

Example 1

Pre-incubation Experiment.

The first example demonstrates that both the energy source and the amino acid source are being depleted. Throughout the examples to follow, two standard methods were used for cell-free protein synthesis. The first uses phosphoenol pyruvate (PEP) and pyruvate kinase to regenerate ATP from ADP. This is termed the PEP system. The second uses pyruvate and pyruvate oxidase to regenerate ATP. This is called the pyruvate system.

The standard reaction mixture for the PEP system produces a base level of protein expression (approx. 100 µg/ml or 13.8 µg/mg cellular protein) and consists of the following components:

57 mM Hepes-KOH (pH 8.2), 1.2 mM ATP, 0.85 mM each of GTP, UTP and CTP, 1mM DTT, 0.64 mM cAMP, 200 mM potassium glutamate, 80 mM NH₄(OAc), 15 mM Mg(OAc)₂, 34 mg/ml folinic acid, 6.7 µg/ml plasmid, 33 µg/ml T7 RNA polymerase, 500 µM

each of 20 unlabeled amino acids, [^3H] leucine (0.27GBq/mmol), 2% PEG 8000, 20 mM PEP (phosphoenol pyruvate), and 0.24 volumes of S30 extract.

T7 RNA polymerase was prepared from the culture of *E.coli* strain BL21 (pAR1219) according to the slightly modified procedures of Davanloo *et al.* (1984) Proc. Natl. Acad. Sci. U.S.A. **81**: 2035-2039. S30 extract was prepared from wild type *E.coli* K12 (strain A19) according to the procedures reported earlier (Kim *et al.*, *supra.*). The standard reaction mixture for the pyruvate system is nearly identical. The PEP is omitted and in its place is added: 33 mM pyruvate, 6 U/ml pyruvate oxidase, 6.7 mM potassium phosphate, and 3.3 mM TPP (thiamine pyrophosphate).

Reactions were conducted for given time periods in a water bath set at 37°C. The amount of synthesized protein was estimated from the measured cold TCA-insoluble radioactivities as described (Kim *et al.*, *supra.*) Radioactivities of samples were measured in a liquid scintillation counter (Beckman). SDS-PAGE analysis was carried out using a 16% SDS-PAGE gel (NOVEX) with standard molecular weight markers. Expressed proteins were visualized by standard Coomassie Blue staining method.

Early experiments had indicated that protein synthesis stopped with the PEP system after about 30 minutes, and after about 200 minutes for the pyruvate system. However, calculations suggested that insufficient protein had been synthesized to account for depletion of the required reagents. To investigate the cause of synthesis cessation, pre-incubation experiments were employed. In these experiments, no DNA template was added and the measurement of radioactive leucine incorporation showed that insignificant protein synthesis occurred. The pre-incubation was conducted for various time periods after which the DNA template was added and protein synthesis measured after a one-hour incubation period. The results in Figure 1 show that the ability of the reaction mixture to support protein synthesis rapidly deteriorated even when no protein was expressed. Adding more energy source could not restore activity; neither could more amino acids. Activity could only be restored by a second addition of the energy source, PEP, and the 20 amino acids.

These surprising results showed that both the energy source and the amino acids are inactivated independent of protein synthesis.

Example 2

Supplementation Experiments.

In another set of experiments, no pre-incubation was used. Instead repeated additions were made to the synthesis reaction mixture in an attempt to prolong the reaction. The results of such an experiment with the pyruvate system are shown in Figure 2. The

initial volume of each reaction was 15 μ l. At each hour after the reaction was started, 1.1 μ l was added. In Control 1, water was added; in Control 2, 0.25 μ l of 2M pyruvate and 0.85 μ l water; in Control 3, 0.85 μ l of amino acid mixture and 0.25 μ l of water; and for the curve labeled "Pyr/AA", 0.25 μ l of 2M pyruvate and 0.85 μ l of amino acid mixture. The curves show the protein synthesis yield per mg of protein in the S30 cell extract. Clearly, the addition of more energy source alone is not sufficient for effective continuation of protein synthesis.

Example 3

Measuring Changes in Amino Acid Concentrations.

Surprisingly, some amino acids concentrations are increasing while others are decreasing much more rapidly than the rate of incorporation into new protein. The observations indicated in the previous examples prompted the measurement of individual amino acid concentrations during the course of a protein synthesis reaction. In this case, a pyruvate-driven reaction of 900 μ l was started. Samples of 100 μ l were taken each hour, diluted 1:1 with cold water and deproteinized by adding 200 μ l of cold 10% TCA (trichloroacetic acid) solution. After centrifugation the supernatant was analyzed using a Beckman 1600 amino acid analyzer. Most amino acids did not change in concentration. However, two very surprising types of observation are shown in Figures 3A and 3B. Alanine and aspartic acid/asparagine both increased significantly in concentration. In contrast, the concentrations of arginine, tryptophan and cysteine all decreased significantly. In the first case, the data suggest that pyruvate is being used for amino acid synthesis rather than for ATP regeneration. This is a potentially serious source of reaction inefficiency. In the second case, the depletion of the three amino acids is most likely the reason that prolonging the reaction required amino acids addition.

Example 4

Supplementation with Arginine, Cysteine and Tryptophan.

Supplementation with only 3 amino acids produces approximately the same amount of product as with supplementation with all 20 amino acids. To test the hypothesis that the depletion of arginine, cysteine and tryptophan was limiting the protein yield, experiments were conducted in which only one or two of the three was repeatedly added. The addition of all three amino acids was required for maximal product yield. Surprisingly, the data in Figure 4 indicate that the addition of only those three amino acids was as effective as the addition of all 20. The experiment was conducted using the PEP system and beginning with 15 μ l of

reaction mixture. Additions were made every 20 minutes. With Control 1, 1.15 µl of water was added every 20 minutes; for Control 2, 0.3 µl of 1 M PEP and 0.85 µl of water; for "PEP/Total AAmix", 0.3 µl of 1M PEP and 0.85 µl of amino acid mixture; and for "PEP/Arg,Cys,Trp", 0.3 µl of 1M PEP and 0.75 µl each of 10 mM arginine, 10 mM cysteine and 10 mM tryptophan solutions.

Even though the "PEP/Arg,Cys,Trp" case was diluted somewhat more because of the larger addition volumes, the protein yield is nearly identical to that with all 20 amino acids repeatedly added. These data indicate that significant cost savings can be realized by adding only a subset of amino acids instead of all 20.

Example 5

Spermidine Slows Arginine Depletion

Five mM spermidine significantly slows the disappearance of spermidine, suggesting that the putrescine and spermidine synthetic pathway is responsible for arginine disappearance.

The data shown in Figures 3 and 4 indicate that the degradation of three amino acids, arginine, cysteine and tryptophan is seriously limiting the efficiency and duration of the protein synthesis reaction. Existing knowledge of bacterial physiology suggests that the action of the enzyme, tryptophanase, is a likely cause of both tryptophan and cysteine disappearance. In the case of arginine, one possible cause is the action of arginine decarboxylase. This is the first enzyme in the pathway for the conversion of arginine to putrescine and spermidine, cationic molecules important for normal bacterial physiology. Putrescine and spermidine can also be produced from ornithine, but arginine is preferred when it is available. (See Figure 10) Arginine decarboxylase is known to be feedback inhibited by spermidine. An experiment was therefore conducted to see if spermidine could slow the loss of arginine. The data shown in Figure 5 indicate that 5 M spermidine does, indeed, significantly slow arginine depletion. Thus, arginine decarboxylase is, at the least, a significant cause of arginine loss. These data also show that enzyme inhibitors are effective in avoiding amino acid degradation.

Example 6

Oxalic Acid Slows Rate of Aspartic Acid/Asparagine Production and Increases Protein Production.

Shows that oxalic acid, most likely by inhibiting PEP synthase (pps), slows the rate of aspartic acid/asparagine accumulation and also increases the yield of protein.

The data presented in Figure 3 also show that some amino acids are increasing in concentration. At first, this would seem to be fortuitous, since these amino acids will certainly not be depleted. However, it was realized that the formation of these amino acids could significantly diminish the energy available for protein synthesis. In the case of alanine, pyruvate is being directly converted in a reaction with glutamate that is catalyzed by alanine-glutamate transaminase. (See Figure 9.) This reaction directly consumes the energy source in the pyruvate system.

With aspartic acid and asparagine formation, the consequences are even more serious. These amino acids are formed from oxaloacetate which, in turn, is derived from PEP (Figure 9). For the PEP system, this is a direct drain of the energy source. For the pyruvate system, the formation of these amino acids indicates that pyruvate is being converted to PEP. This will decrease the amount of pyruvate available for the pyruvate oxidase reaction. However, even more serious is the realization that this conversion requires the conversion of ATP to AMP. For every mole of PEP that is generated and used for amino acid synthesis, two moles of ATP and one mole of pyruvate are lost.

The conversion of pyruvate to PEP is catalyzed by the enzyme, phosphoenol pyruvate synthetase (pps). Oxalic acid has been reported to inhibit pps. To test to see if the pps reaction is indeed responsible for aspartic acid and asparagine synthesis and to test if these reactions are reducing the protein yield, 2.7 mM oxalic acid was added to both the pyruvate system and the PEP system. Figure 6 indicates that the formation of aspartic acid and asparagine is indeed reduced by this inhibitor. Figures 7 and 8 show that oxalic acid increases the protein yield in both systems. With the pyruvate system, less pyruvate and ATP are being lost to amino acid formation. In the case with PEP as the energy source, pyruvate is the direct product after PEP transfers its phosphate to ADP. That pyruvate will then be converted back into PEP by pps, but at the cost of two ATP's for each new PEP molecule. Thus, even with PEP as the initial energy source, the enzyme, pps wastes ATP. With both pyruvate and PEP as energy sources, inhibiting pps with oxalic acid provided a significant benefit by increasing product yield.

Example 7

Avoiding The Production of Deleterious Enzymes

The previous data indicate that the presence of activities such as those of tryptophanase, arginine decarboxylase and phosphoenol pyruvate synthetase have serious negative effects on protein yield for a cell-free reaction. Using enzyme inhibitors is one effective means to control these reactions. However, the cost of the inhibitors can be

avoided is the enzymes are not present in the cell extracts. One way to avoid them is to grow the cells in a manner, which avoids the induction of these enzymes. One example is to grow the cells with glucose as the major carbon and energy source. The production of tryptophanase is known to be repressed by glucose catabolite repression. Also, because gluconeogenesis is no longer required, the induction of pps, an enzyme primarily used for gluconeogenesis, is likely to be less. Another example is to grow the cells in a defined or semi-defined medium such as those known in the art, but which medium does not contain arginine. In this case, putrescine and spermidine are derived from ornithine and arginine decarboxylase activity will not be induced.

Example 8

Protein Synthesis using Pyruvate as Energy Source

A new approach for the regeneration of ATP during cell-free protein synthesis was developed to prolong the synthesis reaction, by avoiding the accumulation of inorganic phosphate. This approach is demonstrated in a batch system derived from *Escherichia coli*. Contrary to the conventional methods in which exogenous energy sources contain high-energy phosphate bonds, the new system was designed to continuously generate the required high-energy phosphate bonds within the reaction mixture, thereby recycling the phosphate released during protein synthesis. If allowed to accumulate, phosphate inhibits protein synthesis, most likely by reducing the concentration of free magnesium ion.

Pediococcus sp. pyruvate oxidase, when introduced in the reaction mixture along with thiamine pyrophosphate (TPP); and flavin adenine dinucleotide (FAD), catalyzed the generation of acetyl phosphate from pyruvate and inorganic phosphate. Acetyl kinase, already present with sufficient activity in *Escherichia coli* S30 extract, then catalyzed the regeneration of ATP. Oxygen is required for the generation of acetyl phosphate and the H₂O₂ produced as a by-product is sufficiently degraded by endogenous catalase activity.

Through the continuous supply of chemical energy, and also through the prevention of inorganic phosphate accumulation the duration of protein synthesis was extended to 2.5 hours. Protein accumulation levels also increased. The synthesis of human lymphotoxin benefits more than that of chloramphenicol acetyl transferase (CAT) since the former is more sensitive to phosphate inhibition. In the expression of CAT, although the initial rate of protein synthesis in the new system was significantly lower than with the conventional system, remarkable improvement in the longevity of the reaction made the protein synthesis yield comparable to the conventional system using phosphoenol pyruvate (PEP).

The benefits of the present system are most striking in the expression of certain proteins particularly sensitive to the concentrations of inorganic phosphate and magnesium. In the case of human lymphotoxin (hLT), whose expression level was strongly dependent on the concentrations of inorganic phosphate and magnesium, the final yield after a 3 hour incubation reached 1.5 times that of the conventional reaction using PEP.

Material and Methods

Phosphoenol pyruvate (PEP) and *E.coli* total tRNA mixture were purchased from Boehringer-Mannheim (Indianapolis, IN). L-[U-¹⁴C] leucine (11.7 GBq/mmol), L-[U-³H] leucine (4.14 TBq/ mmol) and [5,6-³H] UTP were from Amersham Pharmacia Biotechnology (Uppsala, Sweden). All the other reagents were obtained from Sigma (St. Louis, MO). T7 RNA polymerase was prepared from the culture of *E.coli* strain BL21(pAR1219) according to the procedures of Davanloo *et al.* (1984) Proc. Natl. Acad. Sci. U.S.A. **81**: 2035-2039. Plasmid pK7CAT, which includes the bacterial CAT sequence between the T7 promoter and T7 terminator (Kigawa *et al.* (1995) Journal of Biomolecular NMR **6**(2):129-134) was used as a template for CAT synthesis. For hLT synthesis, plasmid pK7LT was constructed by replacing the CAT sequence of pK7CAT with the human lymphotoxin sequence. S30 extract was prepared from *E.coli* K12 (strain A19) as described earlier (Kim *et al.* (1996b), *supra.*)

The standard reaction mixture consists of the following components: 57 mM Hepes-KOH (pH8.2), 1.2 mM ATP, 0.85 mM each of GTP, UTP and CTP, 1 mM DTT, 0.64 mM cAMP, 200 mM potassium glutamate, 80 mM NH₄(OAc), 15 mM Mg(OAc)₂, 34 mg/ml folinic acid, 6.7 µg/ml plasmid, 33 µg/ml T7RNA polymerase, 500 µM each of 20 unlabeled amino acids and [¹⁴C] leucine (11.0 µM) or [³H] leucine (1.2 µM), 2% PEG 8000, 32 mM PEP, and 0.24 volume of S30 extract. In reactions in which pyruvate was used as the energy regenerating compound, PEP was removed from the standard reaction mixture and 32 mM pyruvate, 6 U/ml pyruvate oxidase, 6.7 mM potassium phosphate, 3.3 mM TPP, and 0.3 mM FAD were added. Reactions were run for given time periods in 15 to 60 µl volumes in a waterbath set at 37°C.

The amount of synthesized protein was estimated from the measured TCA-insoluble radioactivities as described earlier (Kim *et al.*, 1996a, *supra.*) Radioactivities of samples were measured in a liquid scintillation counter (Beckman LS3801). For SDS-PAGE analysis, samples were loaded on a 16% SDS-PAGE gel (NOVEX) with ¹⁴C standard markers (Amersham). Kodak XOMAT film was exposed to a dried gel overnight and developed in an automatic X-ray film developer.

Change of mRNA concentration during the incubation was monitored by measuring the TCA-insoluble radioactivity of samples from reactions containing [^3H]-UTP following the same procedures used for estimation of protein synthesis except that the NaOH-hydrolysis step was omitted.

Quantitative analysis of inorganic phosphate was carried out according to the procedures of Saheki *et al.* (1985) Anal. Biochem. **148**:277-281 after minor modifications. 2 μl samples were taken and mixed with 15 μl of 20% SDS solution and 43 μl water. After sequential addition of 670 μl solution A (100 mM zinc acetate, 15 mM ammonium molybdate, pH 5.0) and 170 μl solution B (10% ascorbic acid), each tube was incubated in a 30°C gyratory incubator for 15 min. Finally, the concentration of inorganic phosphate was estimated from the measured OD₈₅₀ of sample and standard curve.

Results

Non-productive degradation of PEP. The concentration of inorganic phosphate in the standard reaction mixture initially containing 32 mM PEP was measured throughout the reaction period of protein synthesis. A standard reaction mixture (control) and reaction mixtures devoid of the indicated components (Figure 12) were prepared and incubated. 2 μl samples were taken at given time points and the concentration of inorganic phosphate was measured as described in Material and Methods. As shown in Fig. 12, the concentration of inorganic phosphate in the reaction mixture increased linearly during the incubation period.

The accumulation of inorganic phosphate took place at the same rate even without addition of plasmid to the reaction mixture, demonstrating that the generation of phosphate occurs at the same rate in the absence of significant protein synthesis. The background level of protein synthesis due to contaminating mRNA and chromosomal DNA is negligible in our system, since the CPM value of a negative control reaction without plasmid DNA was less than 10% of a reaction programmed with pK7CAT.

The non-productive degradation of organic phosphate compounds reduces the amount of PEP available for ATP regeneration and protein synthesis and also increases the accumulation of phosphate. Without S30 extract in the incubation mixture, the accumulation of inorganic phosphate was negligible, suggesting that phosphate accumulation is catalyzed by phosphatase activity present in the S30 extract and is not the result of a spontaneous degradation of organic phosphate compounds (Fig. 2).

After a 60 min incubation, the concentration of inorganic phosphate in the reaction mixture reached 28 mM. As the maximal concentration of inorganic phosphate that can be directly generated from nucleotide triphosphates is only 7.6 mM (2.4 mM from ATP and 5.1

mM from other nucleotide triphosphates), it was clear that a significant amount of PEP was consumed either through direct degradation by phosphatase(s) or by recharging hydrolyzed nucleotide phosphates.

Comparison of inorganic phosphate concentrations in the incubations with or without PEP confirmed that the inorganic phosphate liberation into the reaction mixture was strongly dependent upon the presence of PEP (Fig. 12). Furthermore, neither the final concentration or production rate of inorganic phosphate in the reaction were significantly reduced even when all four nucleotides were removed from the reaction mixture. These results suggest that PEP is a major target of phosphatases and that uncoupled PEP hydrolysis can significantly reduce protein synthesis yields. In fact, the above results indicate that for a 30 minute protein synthesis reaction, as much as 50% of the energy source can be wasted. This will limit ATP regeneration and could explain, at least in part, the early halt of the *in vitro* protein synthesis reaction. However, subsequent PEP addition did not elongate the reaction period significantly. We then focused on the accumulation of inorganic phosphate in the reaction mixture, which could inhibit protein synthesis, most likely through chelation of free magnesium ion.

Example 9

Use of Pyruvate As Energy Source With Phosphate Recycle

Besides PEP, creatine phosphate and acetyl phosphate have been successfully used as energy sources in various *in vitro* protein synthesis systems of eukaryotic and prokaryotic origins. Both of these substrates supported protein synthesis in our system as efficiently as PEP. However, similar to PEP, they lose their high-energy phosphate bond during the incubation with S30 extract. Thus, it appears that as long as one relies on the direct use of phosphate bond energy sources to regenerate ATP, the non-productive depletion of the energy source and accumulation of inorganic phosphate is almost inevitable. To attain an extended reaction period in the batch system, the present invention provides a system that does not require a high concentration of compounds with high-energy phosphate bonds, in other words, a system that can regenerate the high-energy phosphate donor *in situ* as well as ATP.

Pyruvate oxidase(E.C.1.2.3.3) plays an important role in the aerobic growth of lactobacteria by catalyzing the oxidative decarboxylation of pyruvate in several steps. Importantly, in the presence of the cofactors, TPP and FAD, this enzyme catalyzes the condensation of pyruvate and inorganic phosphate to generate acetyl phosphate, which can serve as an energy source in our *in vitro* protein synthesis system. This reaction was tested

to see if acetyl phosphate could be continuously provided while avoiding the accumulation of inorganic phosphate. The inorganic phosphate produced either from protein synthesis or by degradation of acetyl phosphate would be recycled to generate another molecule of acetyl phosphate. The acetyl phosphate would, in turn, regenerate the required ATP. A simplified diagram for this strategy is depicted in Fig. 1B. In addition, since both acetyl phosphate and ATP are continually generated and depleted, the peak concentration of both is likely to be kept low enough to discourage non-productive hydrolysis.

Expression of CAT in the pyruvate oxidase system. To examine this scheme under the reaction conditions for *in vitro* protein synthesis, the required compounds were introduced into the reaction mixture at different concentrations and incubated at 37°C for one hour. Standard reaction mixtures containing different concentrations of pyruvate, pyruvate oxidase, FAD, TPP, and inorganic phosphate were prepared and incubated for an hour. The final amount of [³H] leucine incorporation was measured as described in Materials and Methods. Figure 13A; 6.7 U/ml pyruvate oxidase, 0.3 mM FAD, 3.3 mM TPP, 6.7 mM inorganic phosphate. Figure 13B; 32 mM pyruvate, 0.3 mM FAD, 3.3 mM TPP, 6.7 mM inorganic phosphate. Figure 13C; 32 mM pyruvate, 6.7 U/ml pyruvate oxidase, 3.3 mM TPP, 6.7 mM inorganic phosphate. Figure 13D; 32 mM pyruvate, 6.7 U/ml pyruvate oxidase, 0.3 mM FAD, 6.7 mM inorganic phosphate. Figure 13E; 32 mM pyruvate, 6.7 U/ml pyruvate oxidase, , 3.3 mM TPP, 0.3 mM FAD.

As shown in Figs. 13A to 13E, TCA precipitable radioactivity demonstrates that the new strategy for ATP regeneration does support CAT synthesis. Initially, the final amount of synthesized protein was lower when compared with a control reaction using PEP. The amount of synthesized CAT increased with increasing concentrations of exogenous pyruvate oxidase and leveled off at 6.6 U/ml pyruvate oxidase. While relatively insensitive to the concentration of FAD, the yield of protein synthesis responded sharply to the concentrations of TPP and inorganic phosphate giving maximal protein synthesis at 3.3 mM and 6.6 mM respectively. The yield of protein synthesis was almost negligible when pyruvate oxidase was removed from the reaction mixture.

120 µl reaction mixtures of each system were prepared and incubated in 37° C waterbath. 10 µl samples were withdrawn every 30 minutes and the [¹⁴C] leucine-labeled radioactivity of protein was measured as described in materials and methods. After a one hour incubation, the final amount of synthesized protein reached at only 50% of that with the PEP reaction. We examined the time course of protein synthesis in this new system and compared it with the PEP system. Strikingly, as shown in Fig. 14, the initial rate of protein

synthesis in the pyruvate system was maintained for over 2 hours, while the reaction with PEP slowed significantly after 20 minutes. As a result, despite the reduced initial rate of protein synthesis, the new system was able to produce protein at a volumetric yield comparable to the conventional system using PEP.

5 The prolonged protein synthesis period was accompanied by a prolonged maintenance of mRNA level. Measurement of radioactivity from [³H]-UTP incorporated in mRNA showed that the amount of mRNA was maintained stably over 100 minutes in the pyruvate-utilizing reaction whereas it decreased exponentially after a sharp, initial peak in the reaction using PEP (Fig. 15). The rapid decrease of mRNA content can be explained by
10 the short half life of mRNA in the *in vitro* protein synthesis system. Due to the early depletion of energy source, the PEP system would not be able to produce new molecules of mRNA to balance the degradation. On the other hand, the continuous supply of ATP supports the sustained production of mRNA in the pyruvate system.

 The generation of acetyl phosphate through this reaction cycle produces such by-
15 products as carbon dioxide, acetate, and hydrogen peroxide. It was observed that the generation of these by-products did not significantly change the pH of the reaction buffer. Moreover, hydrogen peroxide, possibly the most serious by-product, does not seem to poison the system as suggested by the prolonged, constant rate of protein synthesis. Presumably, our reaction system contains enough endogenous catalase activity to avoid
20 hydrogen peroxide toxicity. Addition of exogenous catalase to the reaction mixture did not affect either the rate or duration of protein synthesis.

 Fig. 16 shows the autoradiograph of samples taken from the PEP- and pyruvate-
using reactions, respectively. This result indicates that in both systems, CAT is nearly the only protein synthesized. 5 µl samples of PEP (Fig. 16A) and pyruvate (Fig. 16B) system
25 containing [¹⁴C] leucine were withdrawn at given times and loaded on a gel. After running, the gel was dried and autoradiography was carried out.

Expression of human lymphotoxin in the new system. To further examine the potential of the new system, we applied it to the expression of human lymphotoxin.
30 Unexpectedly, the expression level of lymphotoxin was much higher in the pyruvate system (150% as compared to the PEP system, see Fig. 17). Plasmid pK7CAT and pK7LT were used to produce CAT and LT in a standard PEP (blank column) system or in the new system using pyruvate (filled column). After examining the effect of inorganic phosphate and magnesium ion on the *in vitro* expression of human lymphotoxin, it was found that the

expression level of this protein was more sensitive to changes in inorganic phosphate and magnesium concentrations than was CAT (Fig. 18).

Fig. 18A, CAT and LT were produced using the PEP system with varying concentrations of $\text{Mg}(\text{OAc})_2$; Fig. 18B, to the standard reaction mixture of PEP system, different concentration of exogenous inorganic phosphate were added and incubated for an hour. Open circles and columns, hLT; filled circles and columns, CAT. In other words, while the expression of CAT shows a relatively broad range of optimal magnesium concentration, slight changes of magnesium concentration dramatically affect the yield of hLT. Also, when different concentrations of exogenous inorganic phosphate were added to the reaction mixture, lymphotoxin synthesis was more vulnerable to excess inorganic phosphate.

The findings described here demonstrate that the coupling of the *in situ* generation of an energy source such as acetyl phosphate with the ATP regeneration reaction provides a steady supply of ATP for the protein synthesis reaction. This, in turn, allows the initial rate of protein synthesis to be maintained over greatly extended reaction periods. These results suggest that the early halt of protein synthesis experienced by conventional systems can be at least partly attributed to the degradation of ATP regenerating compounds.

Conventional batch *in vitro* protein synthesis systems require relatively high initial concentrations of PEP (around 30 mM) for maximal yield of protein synthesis. Such a high concentration will increase the rate and extent of non-productive degradation as it allows phosphatases with low affinities for PEP to bind and degrade the energy source. In contrast, the concentration of acetyl phosphate in the new system will not greatly exceed that of exogenously added inorganic phosphate (6.7 mM) although there may be marginal contribution of phosphate released from nucleotide triphosphates (1.2 mM ATP and 0.8 mM each of GTP, UTP and CTP). More than likely, the acetyl phosphate concentration will be much lower since its generation appears to be rate limiting and the reported K_m value of acetate kinase for acetyl phosphate is 0.2 mM (Kessler and Knappe (1996) In *Escherichia coli* and *Salmonella* Cellular and Molecular Biology, Neidhardt *et al*, eds. American Society of Microbiology, Washington, D. C., pp. 199-205). Thus, a steady supply of acetyl phosphate, maintained at a rate limiting concentration, in our system is expected to improve the efficiency of high-energy phosphate bond utilization.

In addition to the depletion of ATP regeneration potential, degradation of PEP leads to a stoichiometric accumulation of inorganic phosphate, which can act as an inhibitor both on the transcription and translation reactions for protein synthesis through sequestration of essential magnesium ion from the reaction mixture.

By employing a method which recycles the inorganic phosphate ion, this problem was averted. In addition, as was shown with the expression of human lymphotoxin, our new system would be most advantageous for proteins whose expression is most sensitive to inorganic phosphate. This variable sensitivity to phosphate and magnesium concentration may also help to explain the need to determine the optimal magnesium concentration for each new protein. It is hoped the use of pyruvate as an energy source will allow more consistent performance from protein to protein.

Finally, this system enables us to easily control the rate of protein synthesis by adjusting pyruvate oxidase activity in the reaction mixture. This will provide a useful method to study various aspects of protein synthesis including protein folding as a function of the rate of protein synthesis.

Our subject system provides economic benefits. As is well known, the high cost of *in vitro* protein synthesis systems has been one of the obstacles preventing its use as a commercial method for protein production. Among the reaction components, the high energy phosphate bond compound contributes the largest fraction of cost. A calculation based on the reagent prices shows that PEP accounts for more than 70% of the total reagent cost. Probably, the cheapest source of commercially available ATP regenerating compound would be acetyl phosphate. However, since the cost for pyruvate is almost negligible compared to acetyl phosphate, not to mention PEP, this system will greatly reduce the cost of reagents for the cell-free production of proteins. Further, the cost for pyruvate oxidase can be eliminated by cloning it in the *E.coli* strain used for the preparation of S30 extract.

Through various efforts to enhance the initial rate of protein synthesis of our present system while maintaining the remarkably extended reaction period, a cell-free protein synthesis system of batch configuration may be developed, which enables economical, multiplexed production of families of bioactive proteins. In addition, such approaches can contribute to the development of new approaches for the economical, large scale production of commercial proteins.

Example 10

Protein Synthesis with Repeated Addition of Energy Source

Synthetic reactions were set up as described in Example 8 and Example 9 for the synthesis of CAT, except that a mixture of PEP and amino acids was added every 20 min. to the PEP system; and a mixture of pyruvate and amino acids was added every hour to the

pyruvate system. In both cases, the same concentration used initially was added each time. The results are shown in Table 1, and Figures 9A and 9B.

	PEP system			Pyruvate system	
	Yield of protein synthesis($\mu\text{g/ml}^{**}$)			Yield of protein synthesis($\mu\text{g/ml}^{**}$)	
Incubation time(min)	Control	PEP/AA*	Incubation time(hr)	Control	Pyr/AA#
0	0	0	0	0	0
20	98	100	1	142	150
40	102	147	2	176	327
60	110	206	3	201	461
80	102	259	4	219	545
100	102	266	5	223	648
120	104	281	6	219	706
140	104	268			

5 * Additions every 20 minutes

Additions every hour

** Yield per ml. of original reaction volume

Clearly, the pyruvate system allows a much longer period of protein synthesis and much higher product yield than the PEP system. Not only is pyruvate much less expensive, the new system also can produce at least twice as much product when extended by repeated additions of amino acids and energy source.

10

WHAT IS CLAIMED IS:

1. A method for enhanced *in vitro* synthesis of polypeptides, the improvement comprising:

synthesizing said polypeptide in a reaction mix optimized for at least one of arginine;
5 tryptophan and cysteine; aspartic acid and asparagine; and alanine metabolism.

2. The method of Claim 1, wherein said optimized reaction mix comprises a metabolic inhibitor of pyruvate metabolism.

10 3. The method of Claim 2, wherein said inhibitor comprises oxalic acid.

4. The method of Claim 1, wherein said optimized reaction mix comprises a metabolic inhibitor of arginine degradation.

15 5. The method of Claim 4, wherein said inhibitor comprises spermidine.

6. The method of Claim 1, wherein said reaction mix comprises an *E. coli* extract deficient in the enzyme arginine decarboxylase.

20 7. The method of Claim 1, wherein said reaction mix comprises an *E. coli* extract deficient in the enzyme tryptophanase.

8. The method of Claim 1, wherein said reaction mix comprises an *E. coli* extract deficient in the enzyme alanine glutamate transaminase.

25 9. The method of Claim 1, wherein said reaction mix comprises an *E. coli* extract deficient in the enzyme *E. coli* pyruvate oxidase.

10. The method of Claim 1, wherein said reaction mix comprises an *E. coli* extract
30 deficient in the enzyme phosphoenol pyruvate synthetase.

11. The method according to any of Claims 6 to 10, wherein said enzyme is genetically inactivated in the *E. coli* source of said extracts.

12. The method according to any of Claims 6 to 10, wherein said enzyme is depleted from said extract by affinity purification.

13. The method according to Claim 1, wherein said reaction mix further comprises a homeostatic secondary energy source and a regenerative enzyme to said synthesis reaction, wherein free phosphate hydrolyzed from ATP is recycled.

14. A method for enhanced synthesis of biological macromolecules, where the synthesis reaction comprises ATP as a primary energy source, the method comprising:

addition of a homeostatic secondary energy source and a regenerative enzyme to said synthesis reaction, wherein free phosphate hydrolyzed from ATP is recycled.

15. The method of Claim 4, wherein homeostatic secondary energy source is pyruvate and said regenerative enzyme is pyruvate oxidase.

16. The method of Claim 15, wherein said pyruvate is present at an initial concentration of at least about 1 mM.

17. The method of Claim 15, wherein said pyruvate is present at an initial concentration of at least about 10 mM.

18. The method of Claim 15, wherein said pyruvate oxidase is present at an initial concentration of at least about 0.5 U/ml.

19. The method of Claim 15, wherein secondary energy sources that result in accumulation of free phosphate are present at an initial concentration of less than about 1 mM.

20. The method of Claim 14, wherein said synthesis of biological macromolecules comprises translation of mRNA to produce polypeptides.

21. The method of Claim 20 wherein said synthesis also comprises transcription of mRNA from a DNA template.

22. The method of Claim 14 wherein said synthesis of biological macromolecules is performed as a batch reaction.

23. The method of Claim 14, wherein said synthesis of biological macromolecules is performed as a continuous reaction.

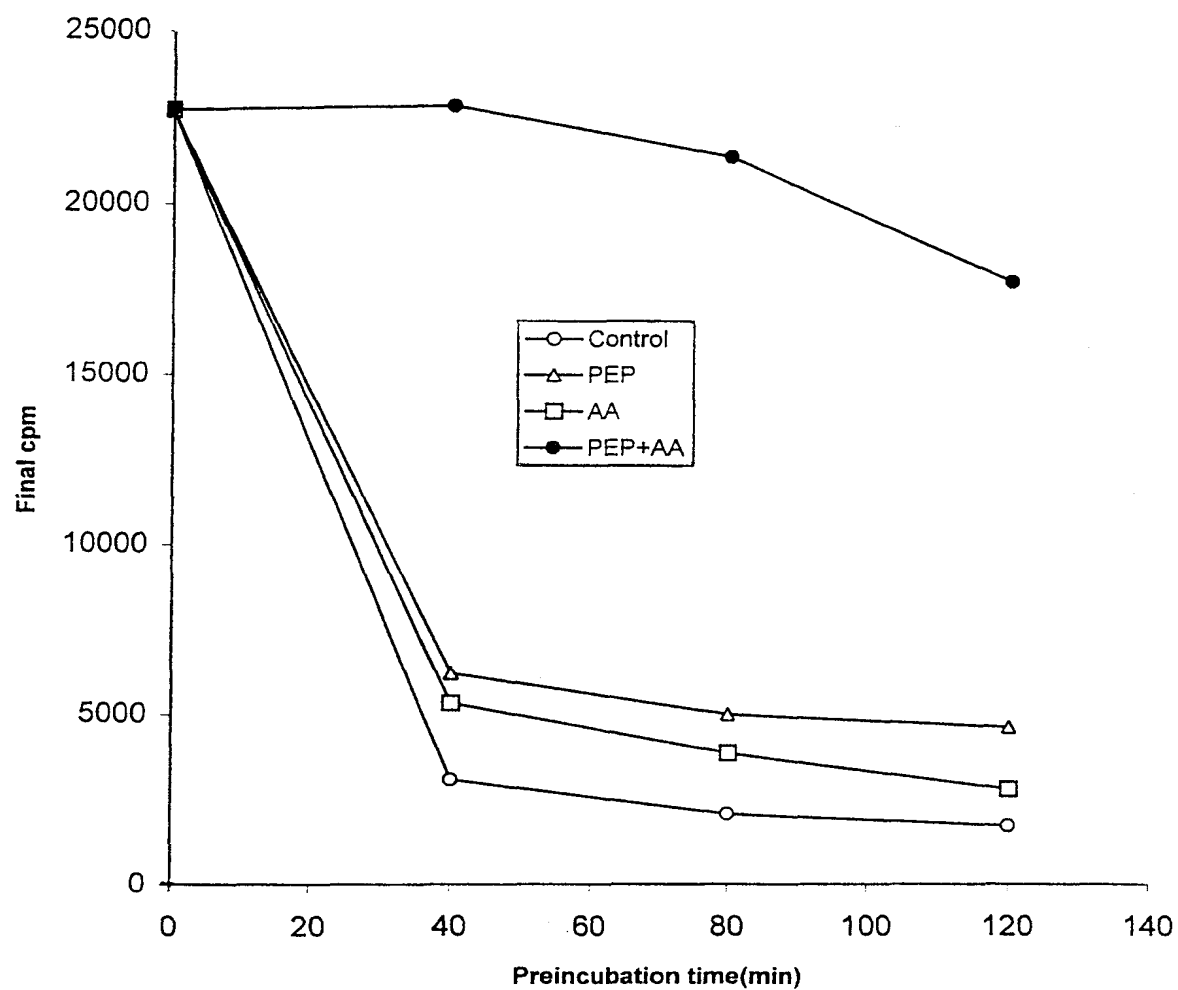
24. The method of Claim 23 wherein said homeostatic energy source is added at
5 intervals during the synthesis.

25. A method for fueling an *in vitro* biological reaction requiring ATP comprising:
addition of a homeostatic secondary energy source and a regenerative enzyme to
said reaction, wherein free phosphate hydrolyzed from ATP is recycled.

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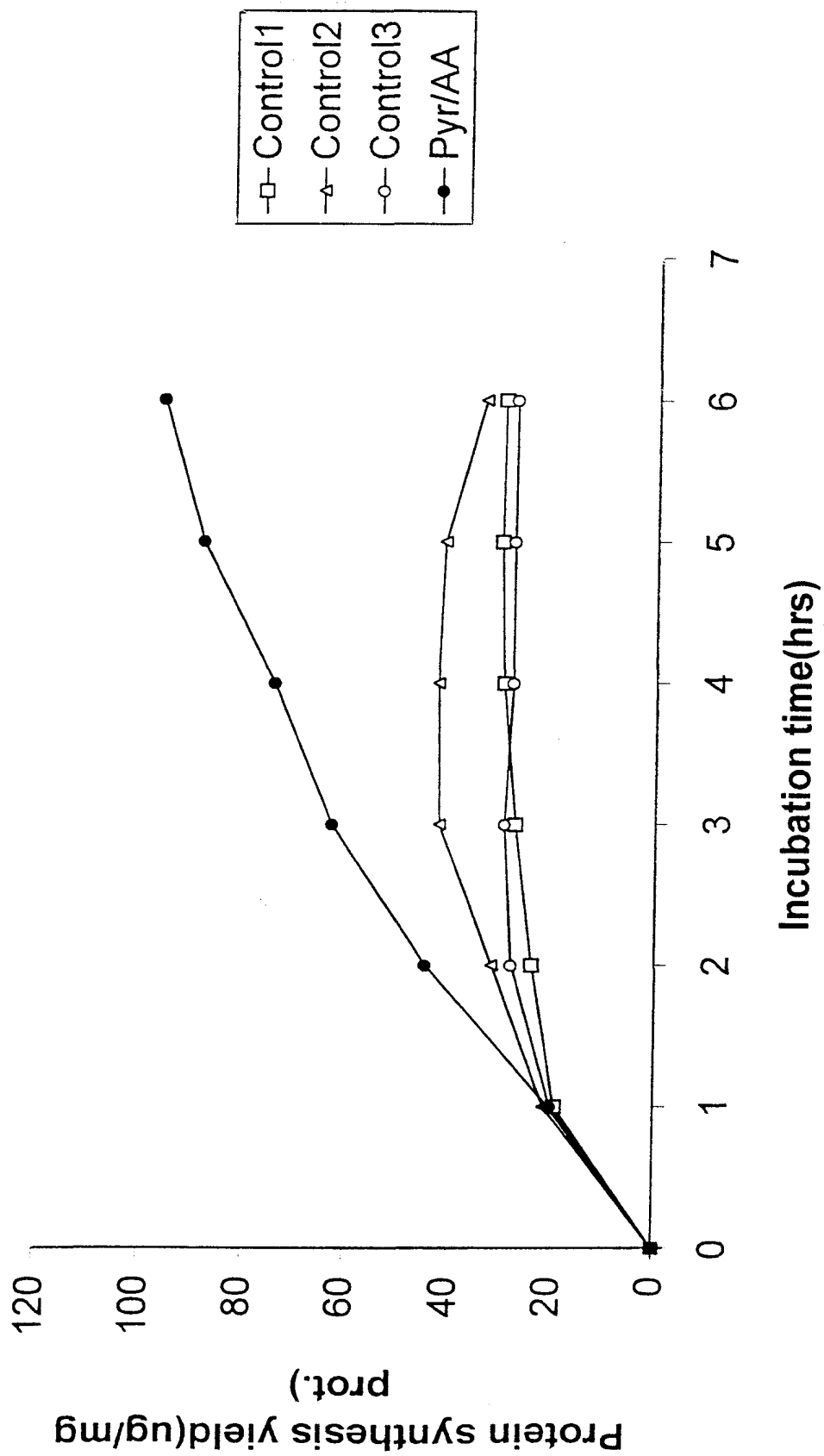
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FIGURE 1
Addition of PEP/AA during preincubation



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FIGURE 2
Supplementation of Pyruvate and Amino acids



Change of amino acid concentration in an E.coli cell-free protein synthesis system
(Pyruvate system)

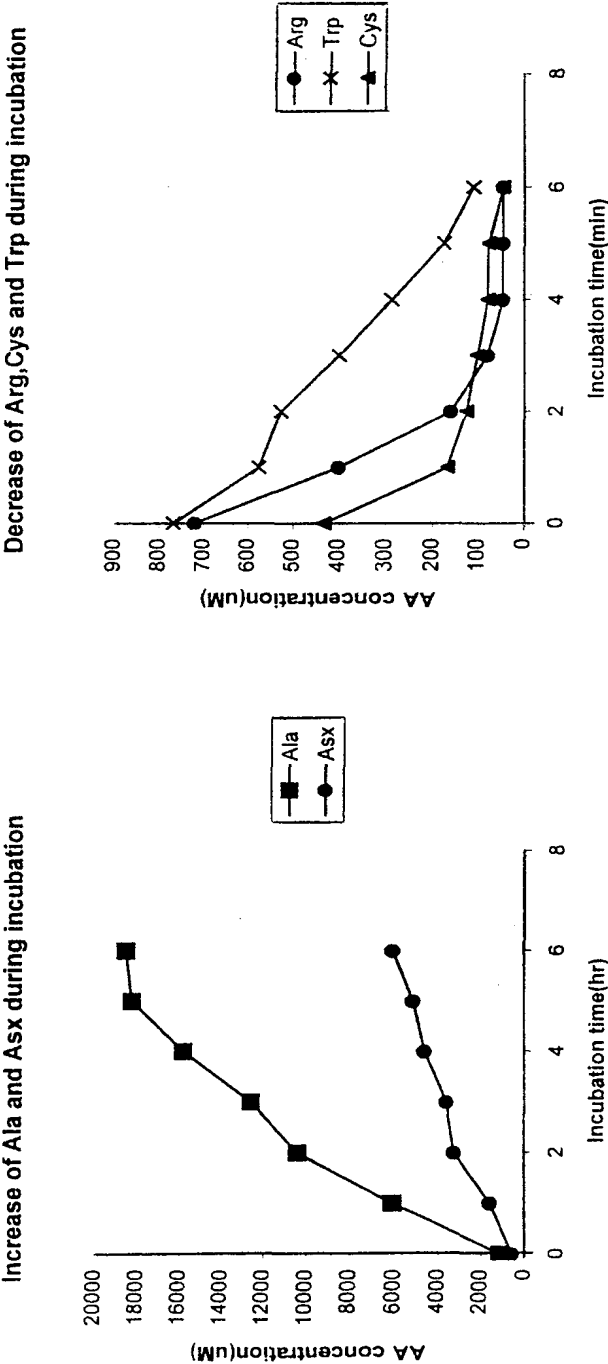
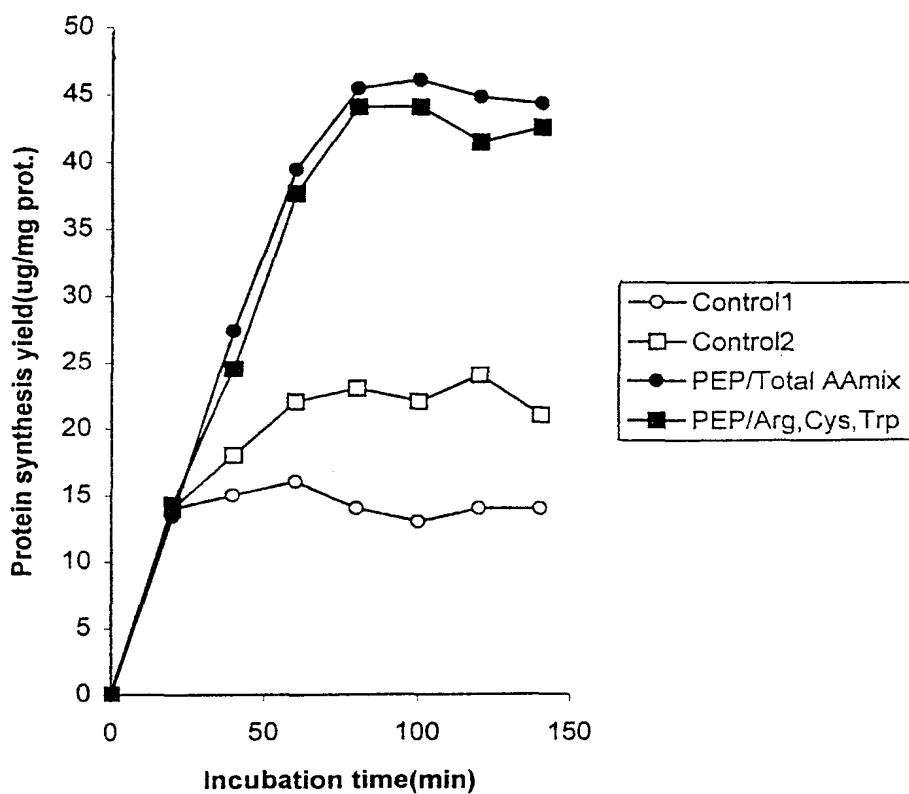


FIGURE 3A

FIGURE 3B

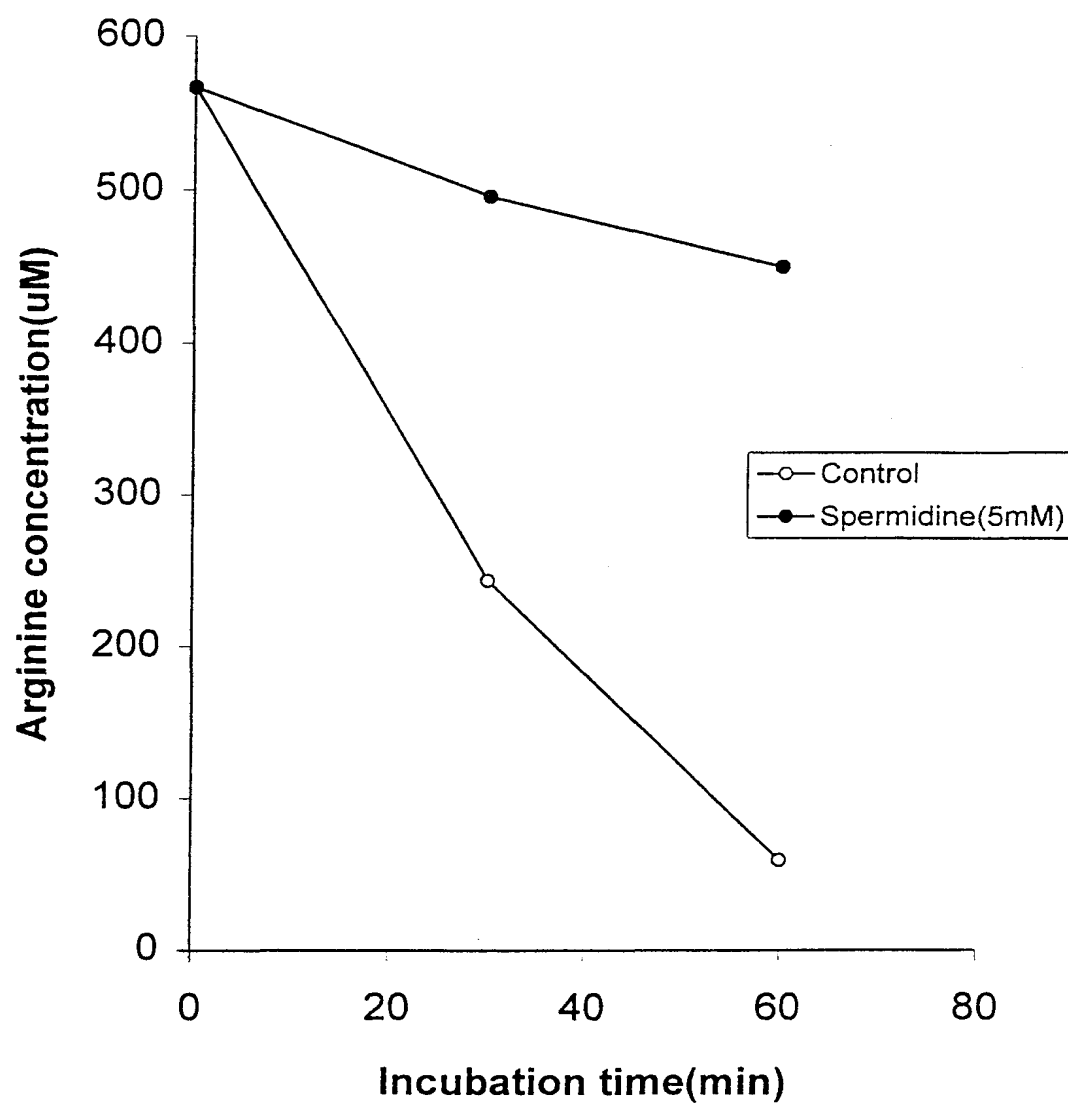
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FIGURE 4
Supplementation of PEP and Amino acids



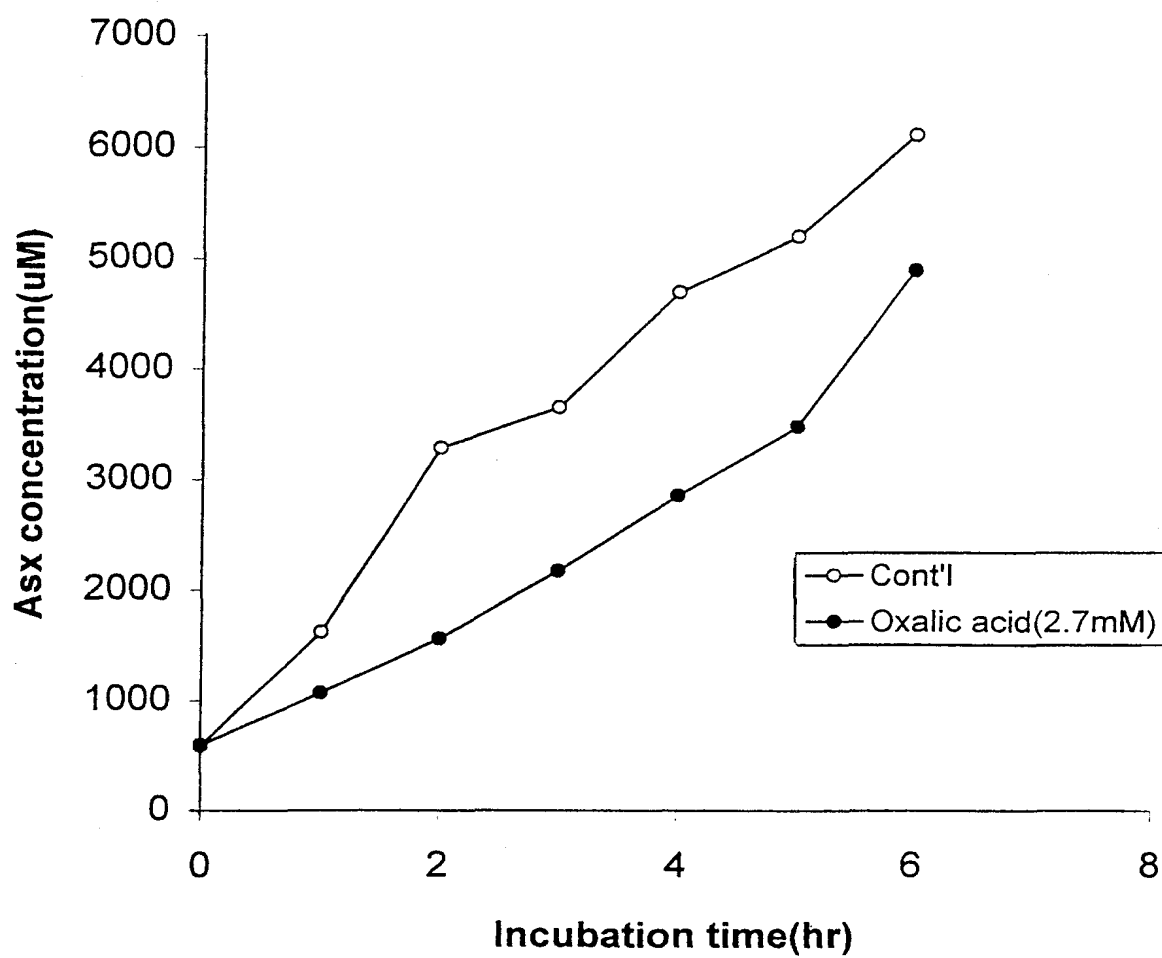
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FIGURE 5



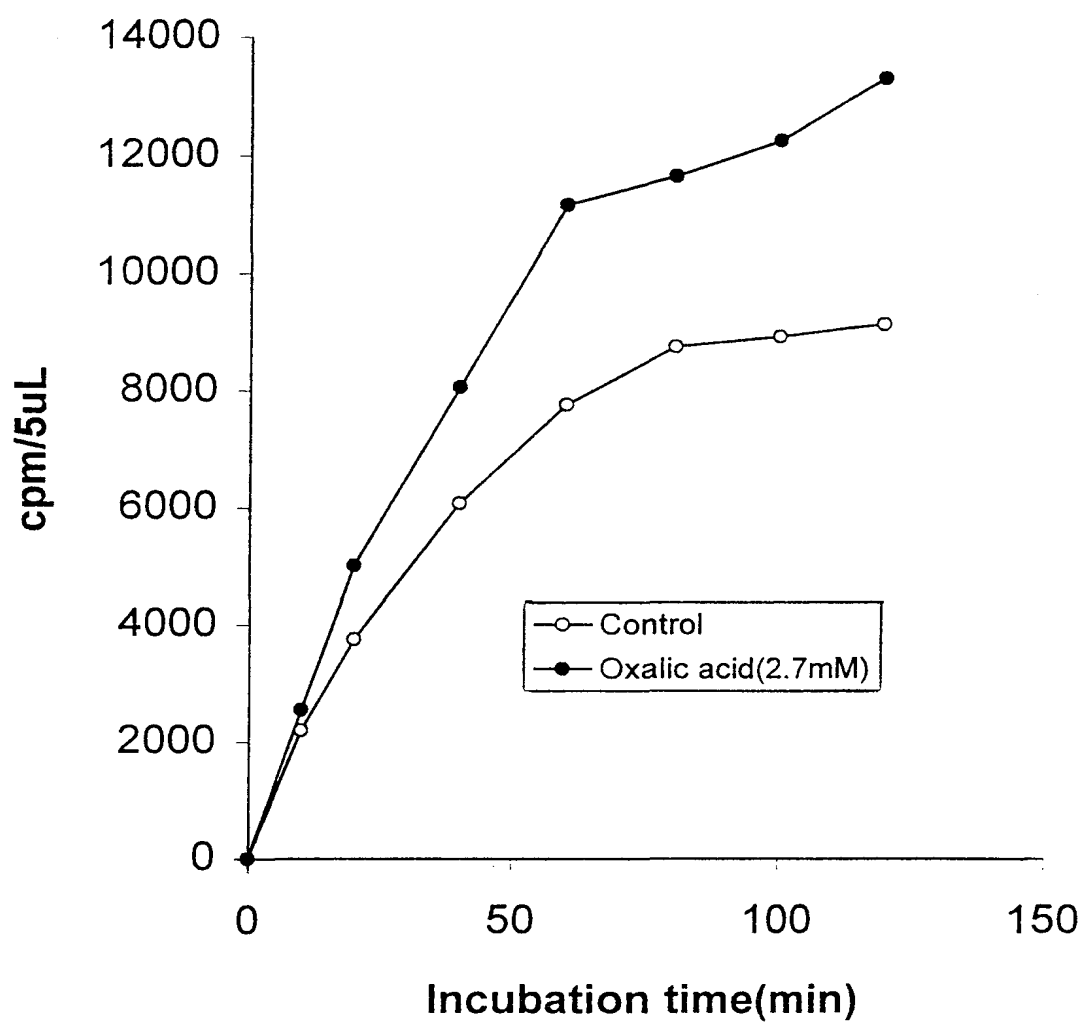
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FIGURE 6
Effect of oxalic acid on Asx generation in the
Pyruvate system



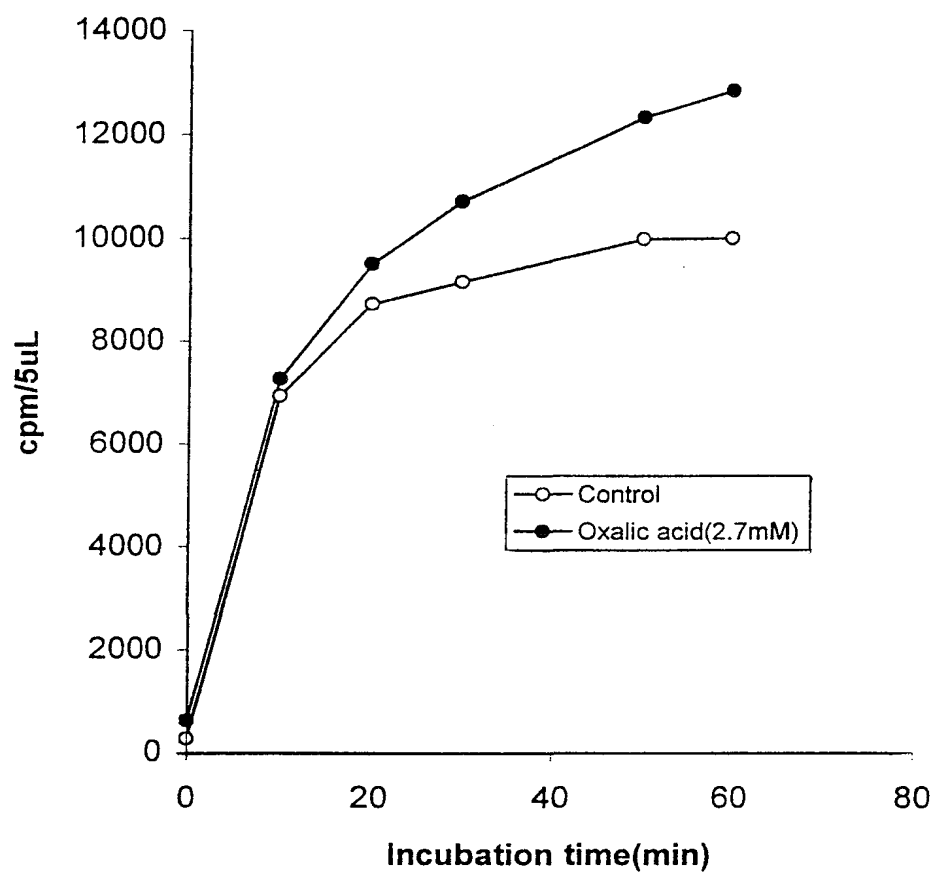
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FIGURE 7
Timecourse of protein synthesis in the Pyruvate system w/ or
w/o Oxalic acid



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FIGURE 8
Timecourse of protein synthesis in the PEP system w/ or
w/o Oxalic acid



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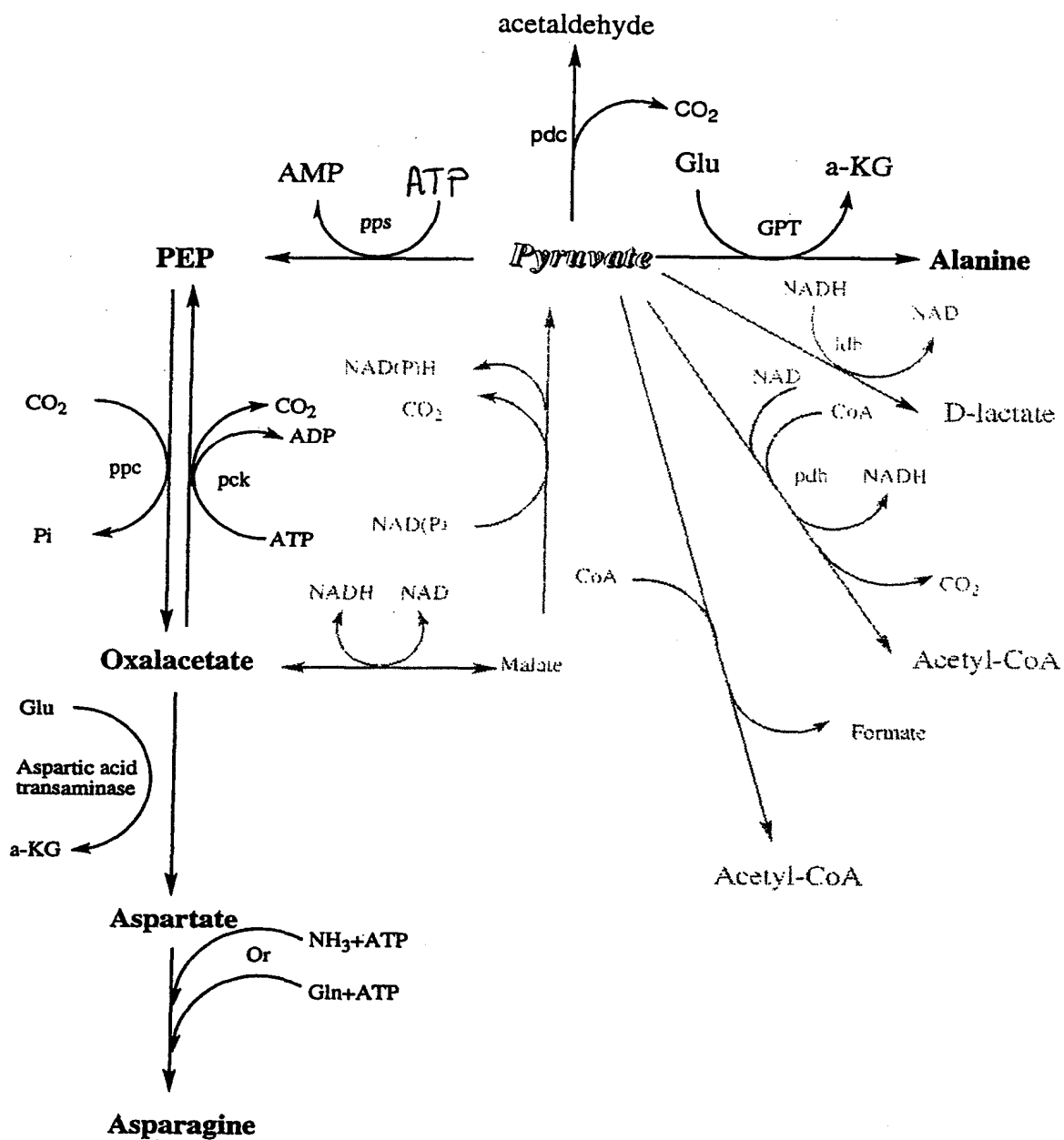
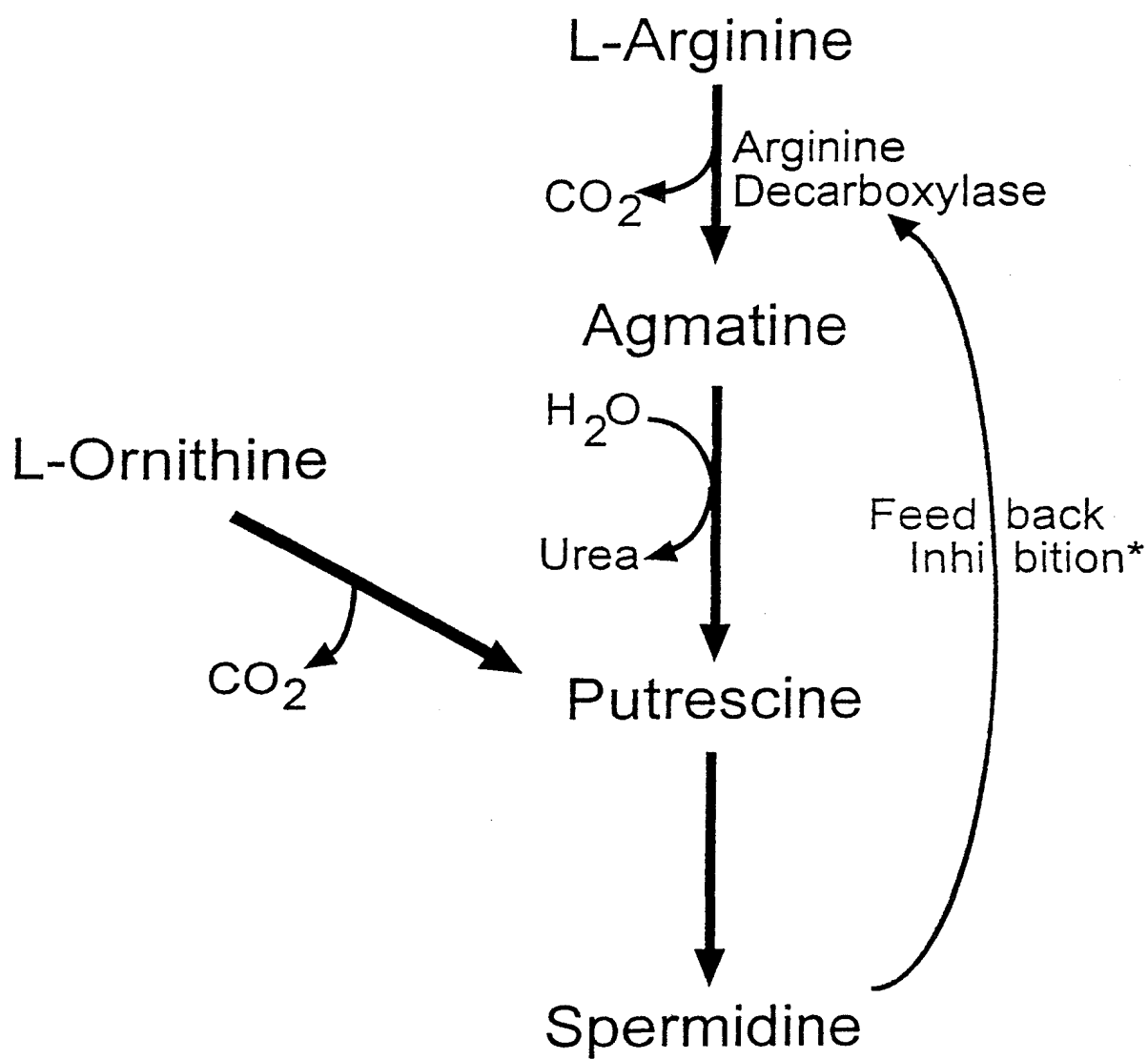


FIG. 9

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Figure 10



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FIGURE 11A

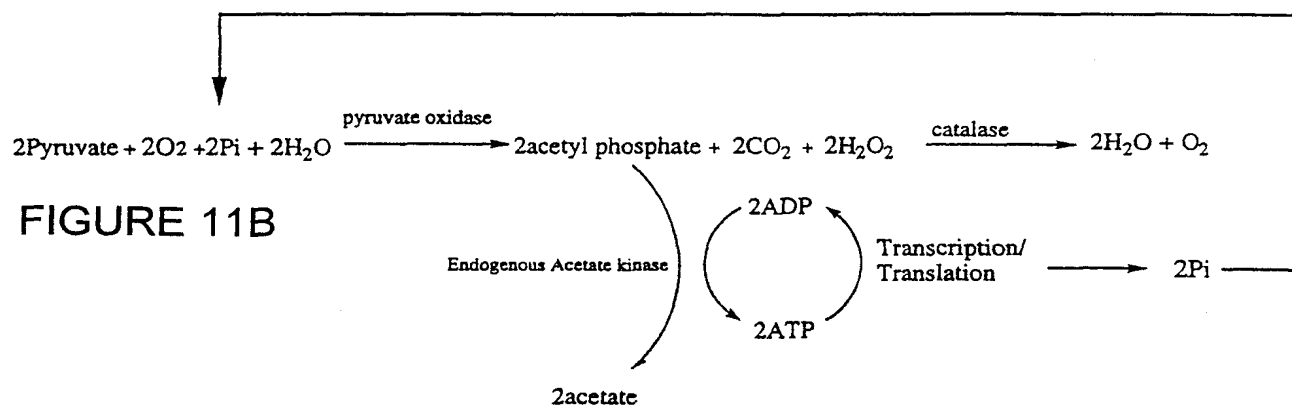
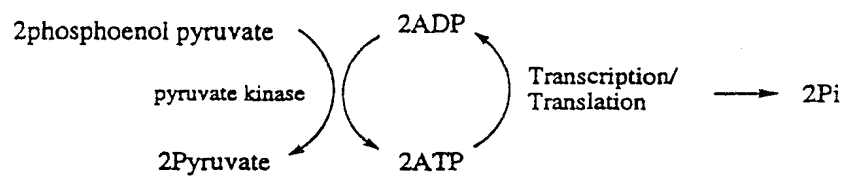


FIGURE 11B

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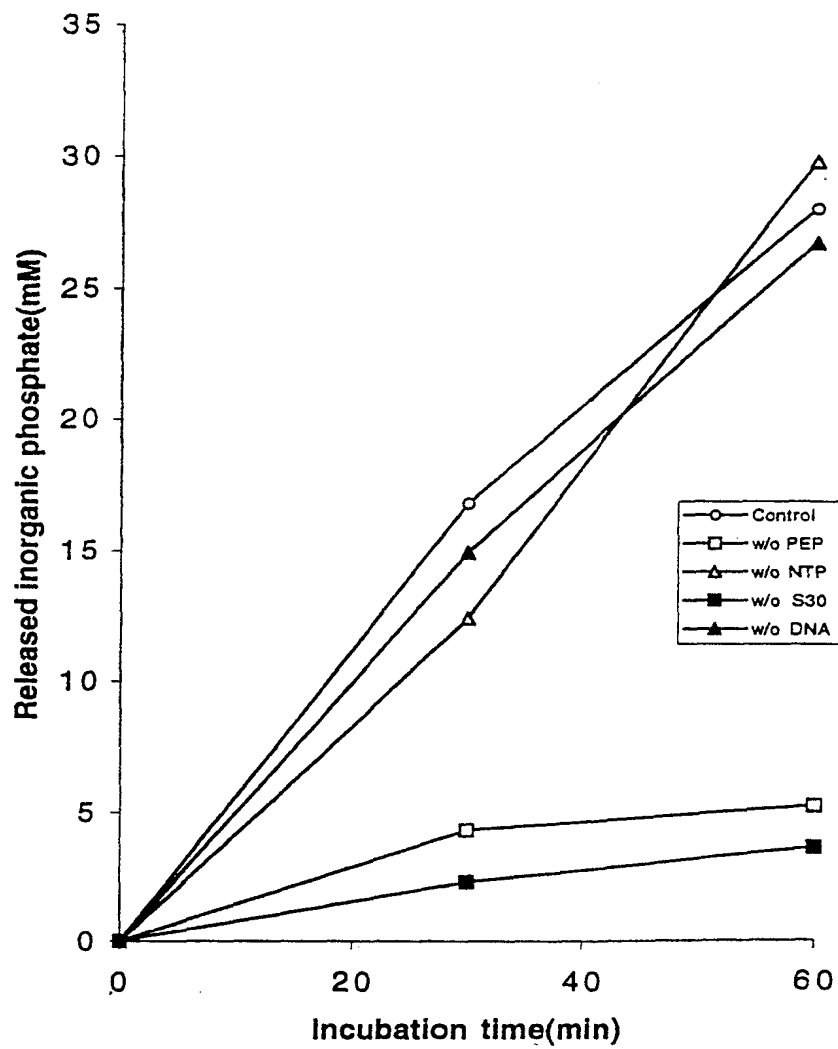


FIGURE 12

FIGURE 13A

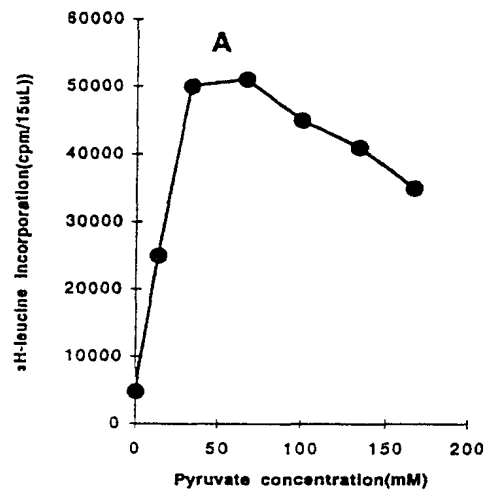


FIGURE 13B

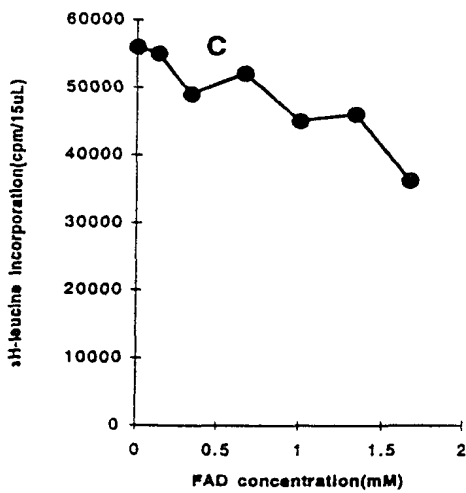
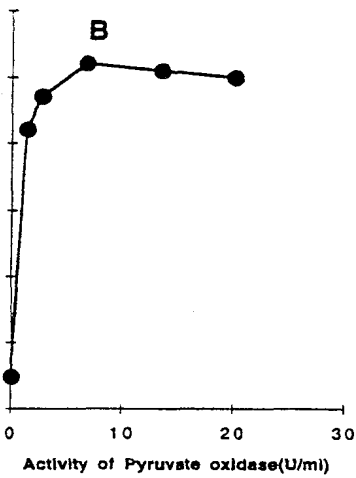


FIGURE 13C

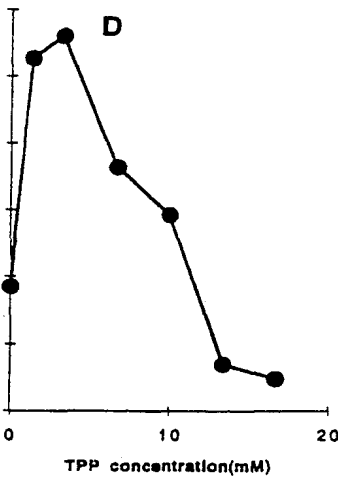


FIGURE 13D

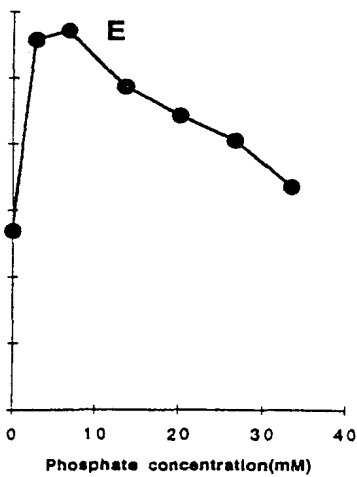


FIGURE 13E

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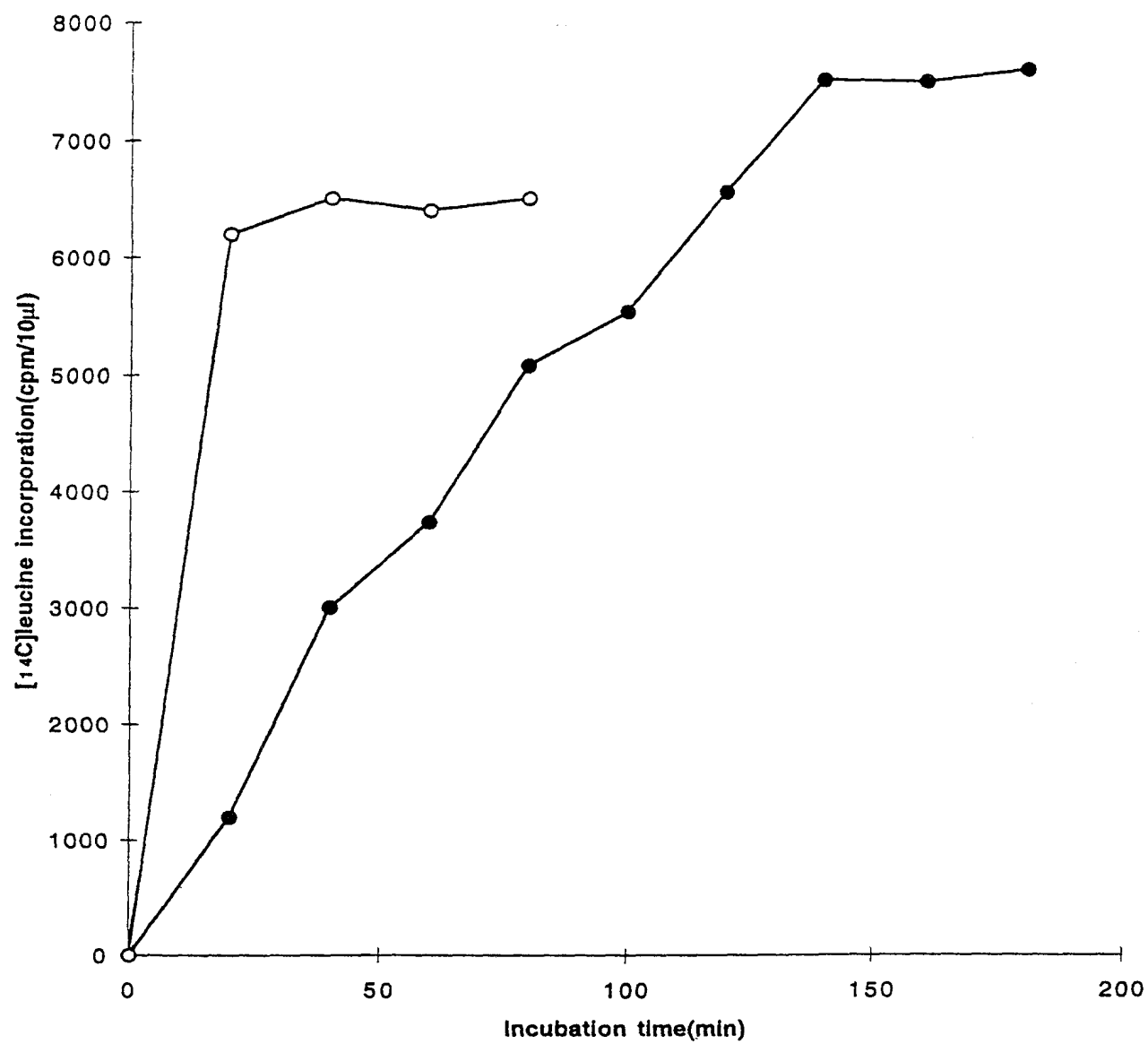


FIGURE 14

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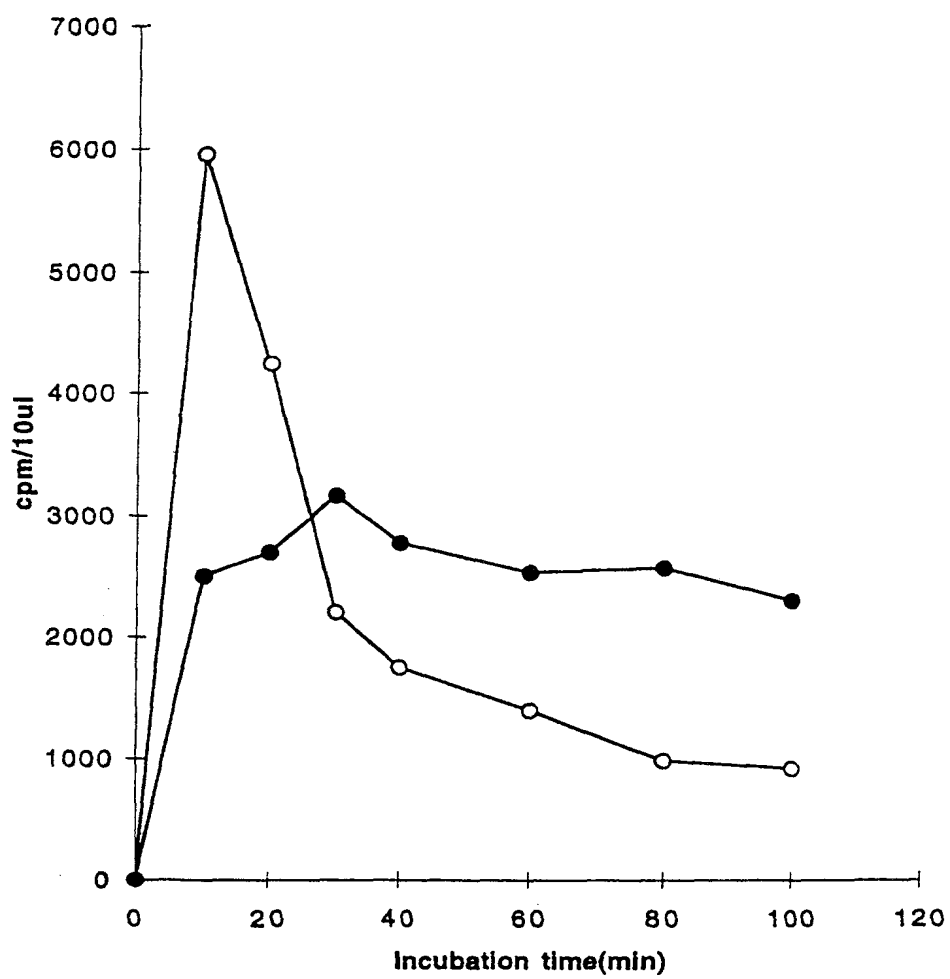


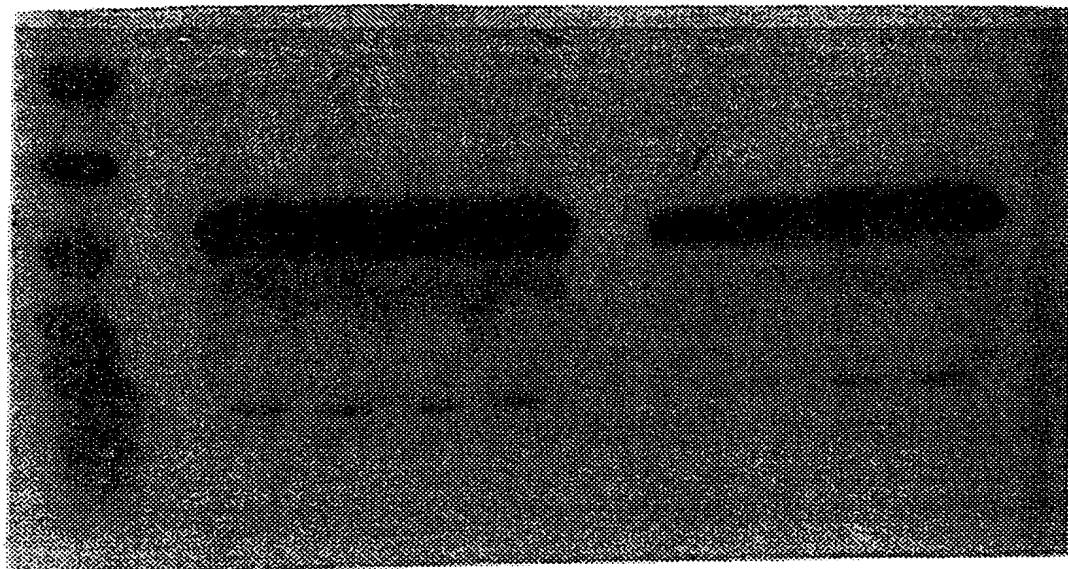
FIGURE 15

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B

M 0 30 60 90 120 0 30 60 90 120 (min)



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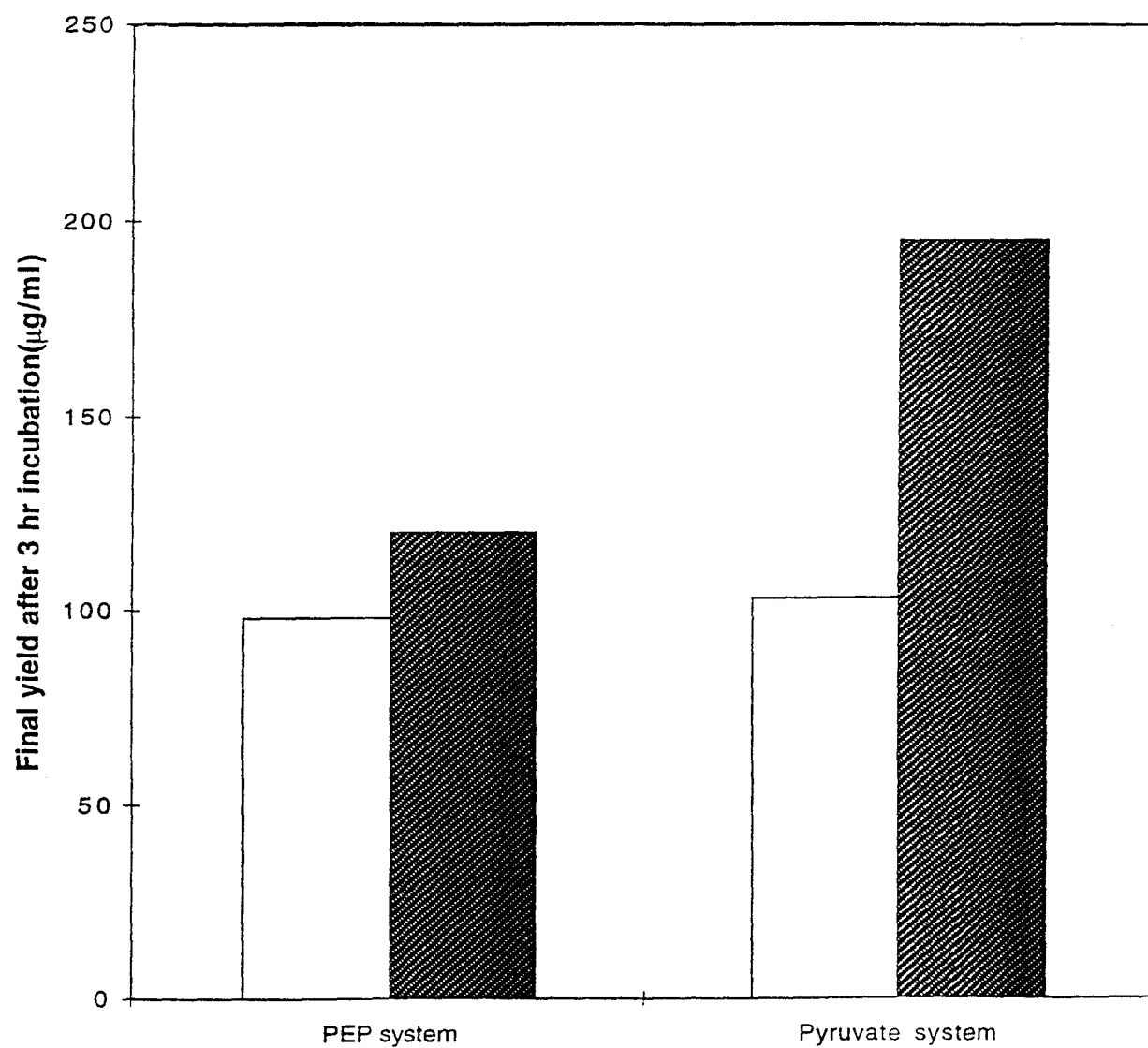


FIGURE 17

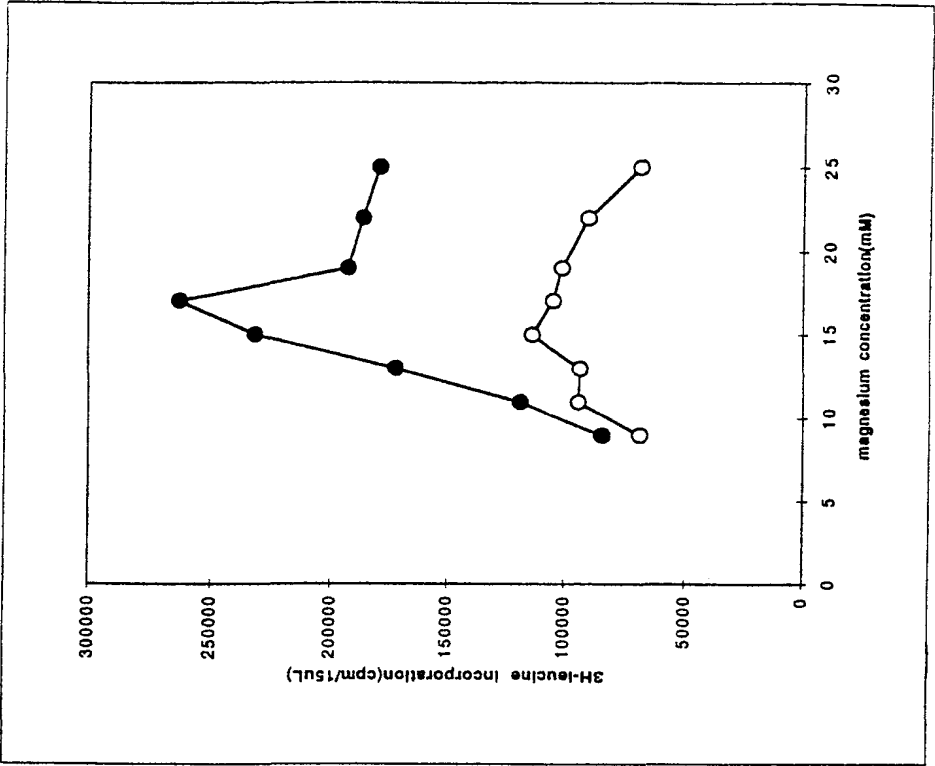


FIGURE 18B

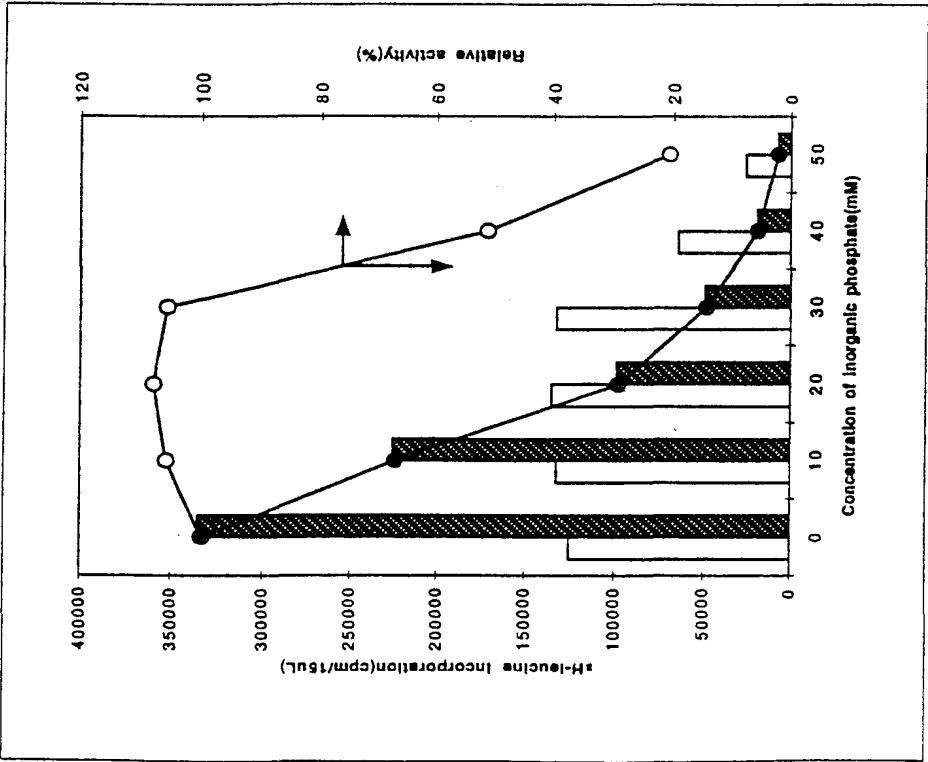


FIGURE 18A

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Repeated addition of energy source/amino acids in a
PEP system

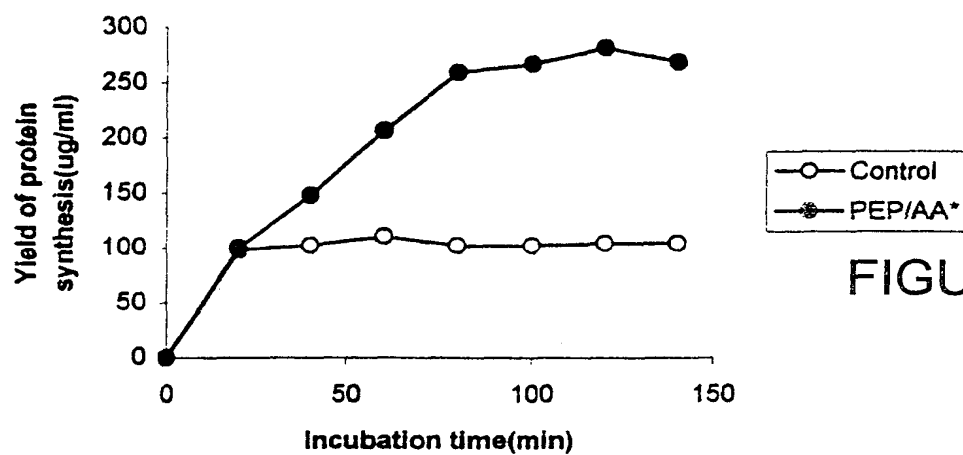


FIGURE 19A

Repeated addition of energy source/amino acids in a
Pyruvate systemamino

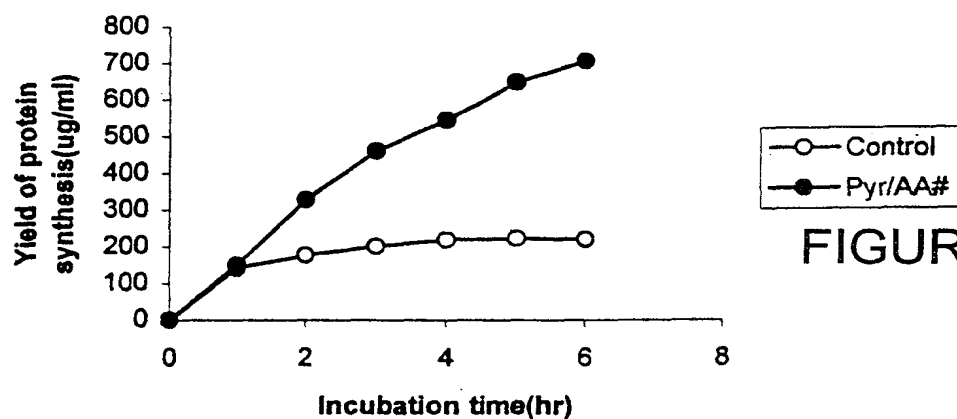


FIGURE 19B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07095

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12P 21/06

US CL : 435/68.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/68.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SNYDER et al. Effects of Polyamine Analogs on the Extent and Fidelity of In Vitro Polypeptide Synthesis. Biochemical and Biophysical Research Communications. 15 May 1991, Vol. 176, No. 3, pages 1383-1392, entire document.	1, 4-5
A	CLEMENS et al. Regulation of protein synthesis in rat liver cell-free systems by amino acids. Proc. Nutr. Soc. December 1972, Vol. 31, pages 273-279.	1
A	PATNAIK et al. Pathway Engineering for Production of Aromatics in Escherichia coli: Confirmation of Stoichiometric Analysis by Independent Modulation of AroG, TktA, and Pps Activities. Biotechnology and Bioengineering. 1995, Vol. 46, pages 361-370.	1, 2, 3, 10, 11

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 09 JUNE 2000	Date of mailing of the international search report 05 JUL 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KATHLEEN KERR Telephone No. (703) 308-0196 JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX <i>[Signature]</i>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07095

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PANAGIOTIDIS et al. Biosynthesis of polyamines in ornithine decarboxylase, arginine decarboxylase, and agmatine ureohydrolase deletion mutants of Escherichia coli strain K-12. Proc. Natl. Acad. Sci. USA. July 1987, Vol. 84, pages 4423-4427.	6, 11
A	CRANS et al. Enzymatic Regeneration of Adenosine 5'-Triphosphate: Acetyl Phosphate, Phosphoenolpyruvate, Methoxycarbonyl Phosphate, Dihydroxyacetone Phosphate, 5-Phospho- α -D-ribosyl Pyrophosphate, Uridine-5'-diphosphoglucose. Methods in Enzymology . 1987, Vol. 136, pages 263-280.	13
A,P	KIM et al. Prolonging Cell-Free Protein Synthesis by Selective Reagent Additions. Biotechnol. Prog. 2000, Vol. 16, No. 3, pages 385-390.	1-12
A	KIM et al. Prolonging Cell-Free Protein Synthesis with a Novel ATP Regeneration System. Biotechnology and Bioengineering. 1999, Vol. 66, No. 3, pages 180-188.	13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07095

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-13

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07095

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN search terms: protein/polypeptide, synthesis/production/expression, cell free/in vitro, spermidine, oxalic acid, arginine decarboxylase, tryptophanase, glutamate transaminase, pyruvate oxidase

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-13, drawn to methods of enhanced in vitro synthesis of polypeptides using reaction mixtures optimized for amino acid metabolism.

Group II, claim(s) 14-25, drawn to methods of enhanced synthesis of biological macromolecules using an homeostatic energy source.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I is the use of reaction mixtures optimized for amino acid metabolism; the claims in Group I all share a technical relationship in that they all require optimization of amino acid metabolism. This technical relationship is not found in Group II, claims 14-25, whose technical feature is encompassed by the use of synthesis reactions containing a homeostatic energy source.